Novel and newer nucleic acid amplification tests for the diagnosis of TB.

By

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DECLARATION

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RESEARCH OUTPUTS FROM THE PROJECT

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Calligaro, G., Theron, G., Peter, J., Meldau, R., ***Matinyenya, B****., et al. (2015). Burden of tuberculosis in intensive care units in Cape Town, South Africa, and assessment of the accuracy and effect on patient outcomes of the Xpert MTB/RIF test on tracheal aspirate samples for diagnosis of pulmonary tuberculosis: a prospective burden of disease study with a nested randomised controlled trial. Lancet Respir Med, 2015. 3(8): p. 621-30.*

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LIST OF ABBREVIATIONS

AFB	acid-fast bacilli
AIDS	Acquired Immunodeficiency Syndrome
AMDT	Amplified *Mycobacterium tuberculosis* direct test
AMV-RT	Avium myeloblastosis virus reverse transcriptase
ART	Anti retroviral therapy
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette Guérin
BIP	Backward inner primer
CDC	Centre for disease control
cDNA	Complementary deoxy-ribonucleic acid
CFU	Colony Forming Unit
CI	Confidence Interval
CRF	Case report form
CSF	Cerebro-spinal fluid
C_T	Cycle threshold
CT	Computerised tomography
DNA	Deoxy-ribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMTD	Enhanced Mycobacterium *tuberculosis* direct test
EPTB	Extra-pulmonary tuberculosis
FDA	Food and Drug Administration
FDR	Flourescent detection reagent
FIP	Forward inner primer
HBV	Hepatitis B virus
HIV	Human Immunodeficiency Virus

IFN-γ	Interferon Gamma
IL	Interleuken
INH	Isoniazid
ICU	Intensive care unit
IP-10	IFN-γ-inducible protein
IQR	Inter-quartile range
IS	Insertion Sequence
LAM	Lipoarabinomannan
LAMP	Loop-mediated Isothermal Amplification
LB	Backward loop primer
LF	Forward loop primer
LJ	Löwenstein-Jensen
LOD	Limit of detection
MAPc	Mycolyl-AG-peptidoglycan complex
MDR TB	Multi-Drug Resistant tuberculosis
MGIT	*Mycobacterium* growth indicator tube
MIP	Monokine-inducible protein
MODS	Microscopic-Observation Drug-Susceptibility
MR	Mannose Receptor
M. africanum	*Mycobacterium africanum*
M. avium	*Mycobacterium avium*
M. bovis	*Mycobacterium bovis*
M. canettii	*Mycobacterium canettii*
M. caprae	*Mycobacterium caprae*
M. intracellulare	*Mycobacterium intracellulare*
M. kansasii	*Mycobacterium kansasii*

M. leprae	*Mycobacterium leprae*
M. microti	*Mycobacterium microti*
M. pinnipedii	*Mycobacterium pinnipedii*
M. tuberculosis	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NASBA	Nucleic acid sequence based amplification
NAATs	Nucleic Acid Amplification Tests
NPV	Negative predictive value
PBS	Phosphate buffer saline
PLG	Phenolglycolipid-lipid antigen
POC	Point-of-care
PCR	Polymerase Chain Reaction
Pol	Polymerase
PPD	Purified Protein Derivative
PPV	Positive predictive value
PTB	Pulmonary tuberculosis
RIF	Rifampicin
RNA	Ribonucleic acid
RPA	Recombinase polymerase amplification
rRNA	Ribosomal ribonucleic acid
SARS	Severe acute respiratory syndrome
SDA	Strand displacement amplification
TB	Tuberculosis
TBM	Tuberculous meningitis
TMA	Transcription mediated amplification
TTP	Liquid culture time to positivity

UCT	University of Cape Town
WHO	World Health Organization
XDR TB	Extremely drug resistant tuberculosis

ABSTRACT

Background: Current tools for TB diagnosis have suboptimal accuracy, perform poorly in diagnosing extra-pulmonary TB, and are not point of care; hence results have a slow turn-around time.

Objective: This project evaluated the diagnostic accuracy of the promising novel loop mediated isothermal amplification (LAMP) assay on sputum, and that of the semi-automated Xpert MTB/RIF (Xpert) test on non-sputum specimens (bronchoalveolar lavage fluid [BALF], tracheal aspirates, and cerebrospinal fluid [CSF]) from South African patients with suspected TB (the accuracy of Xpert using these fluids was unknown at the time this work was performed).

Methodology: Biological samples (sputum, tracheal aspirates, BALF, or CSF) were collected from patients with suspected TB. Liquid culture served as the reference standard for the diagnosis of definite TB. Accuracy was evaluated according to HIV and smear microscopy status, where appropriate. The relationship between test performance and bacterial load (culture time-to-positivity [TTP]) was also compared. For the evaluation of LAMP, 2 spot sputa of approximately 4 ml were collected from 301 patients (60 µl of sputum was used for the assay). For the evaluation of Xpert on BALF, 152 patients who were sputum scarce or smear-negative were recruited (1 ml of the BALF aliquot or a re-suspended pellet from 10 ml BALF was used). For the evaluation of Xpert on tracheal aspirates, 120 tracheal aspirates from patients enrolled in the intensive care unit (ICU) were tested. For the evaluation of Xpert on CSF, 235 patients with suspected TBM had a lumbar puncture with 1 ml of CSF or where available a re-suspended pellet from 3 ml of CSF evaluated using Xpert.

Results: Using sputum samples the sensitivity of LAMP was similar to that of smear microscopy [(77%, 95% CI 67-84%) versus (67%, 95% CI 56-76%; p=0.134), respectively], whereas the specificity of LAMP was suboptimal compared to smear microscopy [(91%, 95% CI 86-94%) versus (99.5%, 97-99%; p<0.003), respectively]. In HIV-infected patients compared to HIV-uninfected patients the sensitivity of LAMP was 70% (54-82%) versus 85% (69-93%); p=0.545, and the specificity was 91% (82-96%) versus 89% (82-93%); p=0.702, respectively, but PPV was similar [(81%, 65-91%) vs. (70%, 55-82%); p=0.291, respectively]. There was a shorter TTP in LAMP-positive patients vs. LAMP-negative patients [16 [IQR: (4-50)] vs. 40 days (6-50);

p<0.0001]. By contrast using BALF, Xpert significantly outperformed smear microscopy [93% (77-98%) versus 58% (39%-74%); p<0.004, respectively]. HIV co-infection was associated with a significantly increased TTP in BALF (23.58 vs. 21.48 days; p=0.02), whilst there was no correlation between Xpert MTB/RIF generated cycle threshold values (C_T) and TTP (p=0.09; R^2=0.654). Centrifugation of the BALF had no incremental diagnostic value having sensitivity of 95% (75-99%) when Xpert was used. Using tracheal aspirates Xpert had a significantly higher sensitivity compared to smear microscopy [91% (62-98%) versus 55% (28-79%); p=0.08]. Using CSF the sensitivity of Xpert was higher than smear microscopy [49% (33-64%) versus 3% (1-15%); p<0.001, respectively]. In HIV-co-infected patients, centrifugation of CSF resulted in a significant increase in the sensitivity of Xpert compared to uncentrifuged CSF [100% (68-100%) vs. 53% (37-69 %); p=0.01, respectively].

Conclusions: In those patients with suspected pulmonary TB, and using sputum, the LAMP assay had incremental yield over smear microscopy, while specificity was suboptimal (< 95%). By contrast when using non-sputum samples Xpert significantly outperformed smear microscopy using BALF in sputum-scarce or smear-negative cases, and using tracheal aspirates in the ICU. In the setting of EPTB, Xpert had good rule-in value for TBM and a higher sensitivity when centrifuged CSF specimens were used.

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SIGNIFICANCE AND OBJECTIVES OF THE STUDY

Study Significance

TB associated deaths account for almost 2 million people yearly. Factors such as a collapse in health standards, coupled with HIVco-infection and a surge in DR-TB have compounded the impact of the disease [1]. Rapid, sensitive, point-of –care molecular TB diagnostic techniques are of significance in eradicating or thus reducing the burden of TB in both resource-poor and resource-advantaged countries. Such techniques should provide a platform that is easy to use and generate results within a short time frame, and should be highly sensitive and provide more specificity than the conventional methods (smear microscopy and culture) currently being used. Furthermore, such techniques should be able to diagnose EPTB and drug susceptibility in patients. So far, the Xpert MTB/RIF test has obtained endorsement from the WHO as a rapid TB molecular platform especially in countries devastated by the disease. In summary, the WHO has outlined the use of Xpert particularly in individuals coming from those areas with greatest risk of the disease having HIV co-infection and/or in individuals from areas with increased risk of MDR-TB. Another promising novel diagnostic tool is the LAMP test, which is a simple and sensitive test for the molecular detection of TB in developing country settings. However, its diagnostic accuracy at the primary level of patient care has not been established yet, such that further research is needed to compare its performance against the conventional modes of diagnosis currently at hand. The results thereof, if positive, will guide in the roll-out of the test especially at point-of-care within primary care settings in developing countries that are at greatest risk of the TB scourge.

Objectives

1. To determine the diagnostic accuracy of the manual LAMP platform on a single spot sputum collected from TB suspects at primary health care centres in Cape Town, South Africa vs. concentrated florescence smear microscopy and liquid culture for the diagnosis of pulmonary TB.
2. To validate the sensitivity and specificity of Xpert in BALF for the diagnosis of sputum scarce and or smear-negative TB.

3. To evaluate the performance outcomes of Xpert using tracheal aspirates in ICU patients for diagnosing pulmonary TB.
4. To determine the diagnostic accuracy of Xpert on CSF in diagnosing TBM.

CHAPTER 1

LITERATURE REVIEW

1.1. TUBERCULOSIS CAUSE, CLINICAL PRESENTATION AND EPIDEMIOLOGY

Tuberculosis (TB) is a respiratory disease falling amongst the top 10 causes of mortality globally [2]. TB mostly affects the lungs and this form of TB is known as pulmonary tuberculosis [3], while EPTB occurs at sites other than the lung, such as meningitis (affecting the central nervous system), Pott's disease of the spine (affecting bones and joints), the lymphatic system (scrofula of the neck), kidneys (urogenital TB) and the skin [4].

1.2. TUBERCULOSIS GLOBAL BURDEN AND PREVALENCE

TB is a global major health threat [2] that has become a global public health epidemic [5]. Of the total world's population, at least one third of the individuals are reported to be infected with *M. tuberculosis* [6] with approximately 2 million deaths occurring on a yearly basis due to TB infection [7-9]. Globally, new TB infection is estimated to be greater than 9 million cases, making it significantly higher than all recorded periods before [5, 7, 10]. The World Health Organization (WHO) reported that in 2008, sub Saharan Africa's estimated incidence rate was believed to be twice that of South-East Asia with over 350 cases per 100 000 population [8].

TB prevalence is greatest in resource poor countries such that approximately 80% of all active TB cases worldwide have been reported to be occurring within such communities [7]. Amongst the new TB cases (approximately 9 million) reported, 15% occurred in HIV-infected individuals where Africa was having 31% from this total reported component [11]. In 2005, the Western Cape Province in South Africa

recorded approximately 1037 cases per 100,000 individuals [12]. TB prevalence has however escalated as a result of the emergence of HIVco-infection amongst other possibilities [12, 13].

1.3. SPREAD OF TUBERCULOSIS

TB is an infectious, respiratory disease caused by *Mycobacterium tuberculosis* complex (MTBC) strains. The mode of transmission is via inhalation of droplet nuclei [8, 14]. *Mycobacterium tuberculosis* (*M. tuberculosis*)-positive patients are highly responsible in the transmission of TB within communities [10] with close contacts suffering the most in terms of getting the disease [15].

1.4. SIGNS AND SYMPTOMS OF TUBERCULOSIS

A persistent cough lasting at least 3 weeks, bloody sputum and chest pain are amongst the most observed signs and symptoms of PTB while weight loss, fever, night sweating, intermittent episodes of fatigue, loss of appetite are also observed signs and symptoms in cases of TB infection in patients [13, 16].

1.5. MULTI DRUG-RESISTANT AND EXTENSIVELY DRUG-RESISTANT TUBERCULOSIS

The widespread misuse of isoniazid together with rifampicin which are first line drugs in the treatment of TB coupled with the emergence of drug resistant TB has resulted in the occurrence of multi drug resistant (MDR) and extremely drug resistant (XDR) TB [9, 17]. MDR-TB is as a result of *M. tuberculosis* that has inherent resistant properties to isoniazid and rifampicin while XDR TB is as a result of *M. tuberculosis* resistant to first-line drugs such as rifampicin and isoniazid, any floroquinolone and either capreomycin, kanamycin and amikacin [18].

Prevalence of drug resistant tuberculosis and global surveillance of DR-TB

About 440 000 MDR-TB incidences occurred globally, with approximately 150 000 mortality cases having been reported in 2008 [17, 18]. India and China had the highest

numbers of reported cases globally contributing at least 50% of the global burden, followed by Russia which had slightly around 9% and sub Saharan Africa with at least 14% of the new global recorded MDR-TB cases, Figure 1 [17].

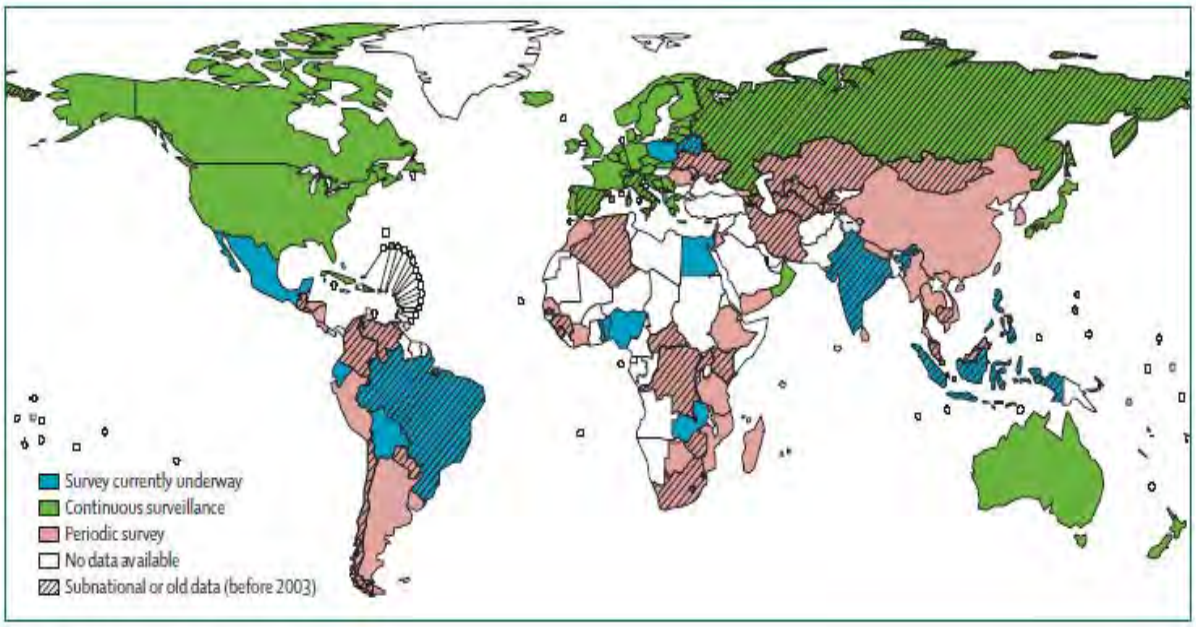


Figure 1: The global coverage and surveillance for MDR-TB prevalence [Adapted from Migliori *et al*.[17]].

1.6. TUBERCULOSIS, HIV CO-INFECTION AND ASSOCIATED DIAGNOSTIC DIFFICULTIES

The escalating TB epidemic is directly related to HIV infection within the general populace [19], especially within Sub-Saharan Africa where the highest TB and HIV rates have been recorded globally [4, 17].

With increased immunosuppression amongst HIV-positive individuals, EPTB and mycobacteremia seems to be highly likely (see Figure 2). This is because T lymphocytes, macrophages and dendritic cells afford the host immune prowess towards TB infection as a result of the various cascade of cytokines that are produced. Th-1 lymphocytes produce interferon-gamma which is central to antimycobacterial immune defence through limiting *M. tuberculosis* growth [12].

In a post-mortem study which was conducted in KwaZulu Natal, Scott *et al.* [20] reported that TB remains a major contributing factor towards mortality especially

amongst HIV-positive patients as a result of the diagnostic delay and possible late treatment regimens in TB patients. HIV infection positively impacts on TB as there is a rather close relationship with an onset of EPTB as a result of the impaired host's inflammatory response due to much lower frequency of cavitation in the lungs, leading to frequent smear-negative TB and an accompanying long time to culture-positivity of liquid cultures [6]. This occurs as the individual at first has proper immunological control and few bacterial numbers (quiescent infection), followed by the progressive disruption of immunological function (active infection) as a result of rising bacillary numbers and the eventual appearance of TB-associated symptoms (see Figure 2) such that active infection is increased by HIV co-infection [5].

With this in mind, employing techniques such as the LAM test, culturing blood and aspirating lymph nodes can be of utmost importance in cases of sputum scarce patients.

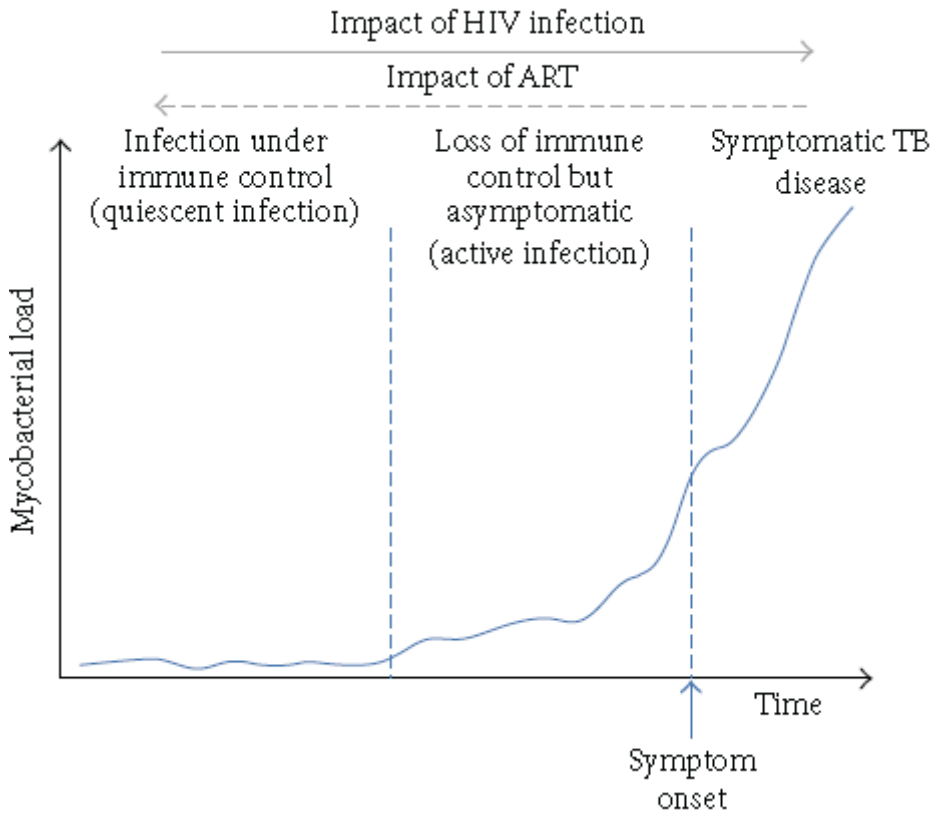


Figure 2: Graph showing the rising total bacillary load of M. tuberculosis over time in an infected patient [Adapted from Lawn *et al*. [6]].

1.7. STRUCTURAL MORPHOLOGY OF MYCOBACTERIUM TUBERCULOSIS

M. tuberculosis is cocco-bacillary in terms of structural morphology with sizes averaging at least 0.6 μm in width and 4 μm in length [21]. The bacterium is an acid fast, obligate aerobe that has limited motility. Organisms form colony-like clumps when in broth culture [22], Figure 3.

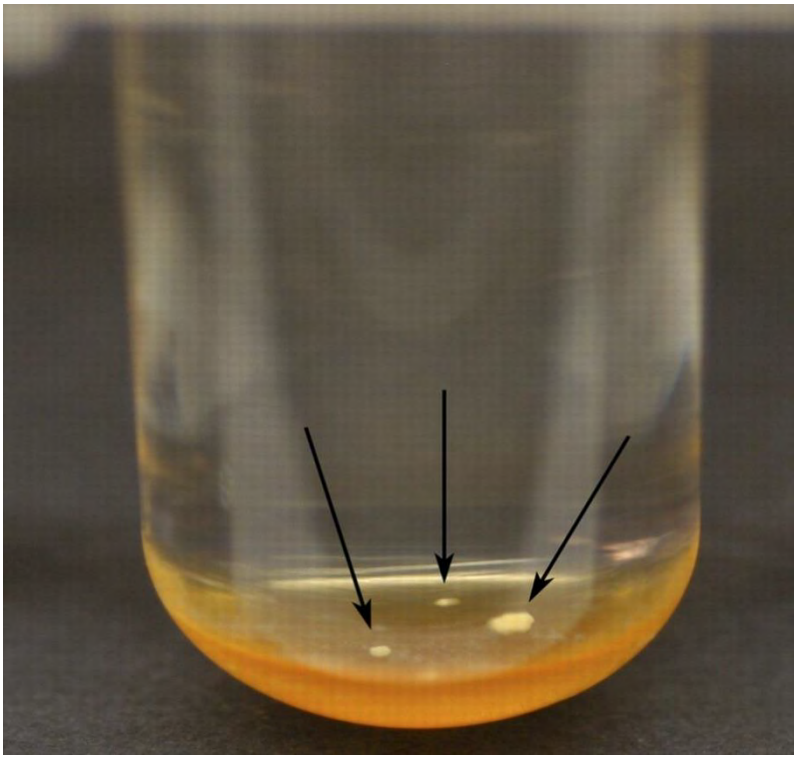


Figure 3: Mycobacterial growth in broth culture [Adapted from Pena *et al*. [22]].

1.7.1. Mycobacterium tuberculosis cell wall constituents

M. tuberculosis is an intracellular parasite that resides within the macrophage. *M. tuberculosis* can neither be classified as Gram-positive or negative species. *M. tuberculosis* has an inner plasma membrane made up of phospholipids, coupled with an outer peptidoglycan layer. Further plasmic membranes do exist, and this gives the bacterium its unique chemical constituents and characteristics in virulence and pathogenesis.

1.7.2. Cell wall composition

The inner plasmic membrane is composed of arabinogalactan, whereas the outer layer has mycolic acid. Approximately, more than 65% of lipids make up the cell wall of the bacterium with mycolic acids, Cord factor and wax-D as the major lipid constituents. Hydrophobic properties are conferred by the mycolic acids on the extracellular layer which thus prevent disinfectants from entering the cell. The mycolyl-AG-peptidoglycan complex (MAPc) forms the core component of this envelope [23]. The markedly observed specific s-shaped cords (chains of cells) especially when *M.* tuberculosis colonies are grown *in vitro*, is as a result of the Cord factor component which is mainly found in virulent strains of mycobacterial species. The extracellular envelope is made up of Wax D which aids in immunogenicity [23].

The biochemical complex formations that are formed between cell surface receptors termed the Mannose Receptor (MR) proteins found on the macrophage and the pathogen are brought about by the interactions of the surface glycolipids [24]. LAM binds to the MR present on the cell surface of the macrophage, thereby causing conformational changes to the structure of the cell wall, inactivating it in the process, creating an immune free system that results in mycobacteremia, while LAM also scavenges for reactive oxygen species that aid in *M. tuberculosis* growth [25, 26]. It is this LAM that molecular assays have been developed for diagnosing TB infection especially within high HIV-positive populations using urine as the bio-sample.

1.8. TB DIAGNOSTICS

1.8.1. Conventional modes of TB diagnosis

1.8.1.1. Microscopy

Sputum smear microscopy and culturing of *M. tuberculosis* especially in marginalized resource-limited countries remain the core TB diagnostic techniques available [27]. Microscopy exists as the primary mode of TB diagnosis in such marginalized set ups [28] and provides an index of contagiousness [27, 29]. Smear microscopy is a relatively simple technique, while it is also inexpensive especially for the general populace who might need the test to be carried out. Amongst its drawbacks is that, in cases of HIV co-infection , disseminated disease and paediatric TB the test is not sufficiently effective, resulting in increased mortality rates due to misdiagnosis [28, 30]. Also the need for experienced professionals that might not be readily available and the need for expensive laboratory-ware further compound's on the test's weaknesses [10].

1.8.1.2. Culture

Culture is the golden standard for diagnosing the presence of MTBC strains while it offers drug susceptibility testing as well [29, 31, 32]. Two types of culture exist, which are solid culture (Lowenstein Jensen) and liquid culture (MGIT liquid culture 960). The major disadvantage culture has is that it is time consuming for both the diagnostic process and drug susceptibility testing, with Lowenstein Jensen (LJ) cultures taking between 20 to 56 days and between 28 to 42 days respectively [31]. HIV-infected individuals and minors find it difficult to expectorate sputum, such that culture will eventually miss EP forms of TB, where EPTB is highly likely in immuno-compromised patients and causes high mortality rates in such populations [31].

1.8.1.3. Radiography

The absence of specific radiological markers associated with TB leads to this technique being prone to errors especially in diagnosing PTB [33]. Misdiagnosis is

highly likely as a result of Chest X-rays which might show normal compatibility, where a patient might actually have active PTB resulting in many false negatives.

Thus the traditional modes of diagnosing TB take long to produce a result and require technical expertise that might not be readily available necessitating the need for novel molecular methods to address these drawbacks.

1.8.2. Antigen-based diagnostic methods

An alternative way of diagnosing TB is through antigen detection. A minimum concentration threshold of at least 20 mg/mL is useful for mycobacterial antigen detection on different body fluids. Amongst the antigens used in the detection of TB are antigenA60, LAM, cord factor, antigen 5, PPD, P32 antigen amongst others [34]. Commercial tests detecting the presence of antigens have been developed for specific bio-samples [31]. However, antigen based methods have the disadvantage of being unable to distinguish between latent and active infection.

1.8.2.1. LAM urine test (Alere, USA)

The assay detects LAM in urine of TB infected patients [31]. LAM forms part of the bacterial cell envelope and confers heat resistant properties to the bacterium, where in TB infected individuals, LAM is ultra-filtered by the kidney and found in their urine [35]. Urine is sterile and thus safe to deal with, while its collection is simple as compared to obtaining sputum or other bio-samples.

Several studies have been carried out on commercial LAM tests using ELISA on urine in detecting TB. In one study, when solid or liquid culture was used as a reference standard, the sensitivity of the test was 51%, while having a specificity of at least 90% [35]. They also reported an increase in sensitivity of about 62% particularly in patients infected with HIV [35]. Similarly, in a study by Peter *et al*. [36], the sensitivity of LAM was 60% in sputum-scarce patients who were HIV-infected.

The use of the LAM test as a solo diagnostic test will not be useful to sufficiently rule-in / rule-out TB due to the modest sensitivities realised. Coupled with smear microscopy, the LAM test can rapidly lead to the identification of new TB cases

especially within HIV-infected populations who are highly immuno-compromised [35].

1.8.2.2. Protein assays (Proteome Systems, Australia) (still under review)

The test being developed has the capability to test on most bio-samples such as blood products (plasma and whole blood), saliva and sputum. Guillerm *et al.* [31], reported that though preliminarily, the test can be able to detect those mycobacterial species specific antigens allowing for active TB diagnosis.

1.8.3. Nucleic acid amplification techniques (NAATs)

Nucleic acid amplification (NAA) testing in clinical medicine is of vital importance, as it allows genetic screening and disease diagnosis [37]. NAATs targeting *M. tuberculosis* have enormous potential to improve TB case detection and thus provide a more timeous result than the existing conventional modes of TB diagnosis together with the biochemical platforms [38]. In culture-positive, smear positive (C^+S^+) patients, NAA testing provides a more sensitive platform in diagnosing TB, while providing with reduced sensitivity within culture-positive, smear-negative (C^+S^-) patients.

1.8.3.1. Commercial nucleic acid amplification tests

Several companies have standardized nucleic acid amplification tests commercially which have widely benefited in the diagnosis of PTB. However, documented studies have rather somewhat variable information on the performance characteristics of these tests and thus indeterminative in comparison to the standard methods for TB diagnosis [39]. It is however imperative that such commercial tests be used in conjunction with microscopy as documented by the US Centre for Disease Control (CDC).

Polymerase chain reaction methods have been established to detect mycobacterial strains [40]. The Food and Drug Administration (FDA) has endorsed use of the Enhanced *Mycobacterium tuberculosis* direct test (E-MTD; Gen- Probe, San Diego, CA) and the Amplicor *Mycobacterium tuberculosis* test (Amplicor, Roche Diagnostic Systems, Inc., Branchburg, NJ) [38, 39, 41, 42], Table 1. The two tests are primarily for the detection of acid fast species in patients who haven't been initiated on TB treatment for a week or thus from the prior year [38].

1.8.3.1.1. Amplicor Mycobacterium tuberculosis test

MTBC organisms possess a specific ribosomal gene, the 16S rRNA which is amplified by specially designed primers [40, 41]. A segment of this gene is amplified, which has a specific code that allows complimentarity with specific oligonucleotides for mycobacterial species. Contamination is limited through the addition of dUTP and a restriction enzyme which can either induce false-positive results [41]. Following the decontamination process by sodium hydroxide, clinicians and patients can expect results in about 6 ½ hrs, Table 1. A computerised system, the Cobas Amplicor is thus available in developed countries. The test has been shown to be highly specific and sensitive in *M. tuberculosis* diagnosis particularly amongst S^+C^+ sputum samples with rather reduced sensitivity in S^-C^+ sputum samples [40]. Clinical evaluation studies of the test have given sensitivity of between 80% - 92%, and specificity of at least 99% [41]. European countries are already using this test, while it is still waiting for endorsement in the USA.

1.8.3.1.2. Enhanced Mycobacterium tuberculosis Direct Test

The test is fundamentally based on amplification of a transcript, whereby the basic principle that starts the process is as a result of a pre-sonication step that disrupts the cell and results in rRNA being released from the bacterial [41]. A complex is formed between the bacterial rRNA and a promoter-primer which initiates amplification. rRNA is reverse transcribed to form a cDNA-RNA hybrid resulting in the primary ribonucleic acid template in being degraded giving rise to promoter number 2 binding to the complimentary-DNA extending it in the process, giving rise to ds-cDNA that has a DNA-dependent RNA polymerase further initiating transcription such that new ribosomal ribonucleic acid molecules are formed [41].

The clinical sensitivity and specificity of this test has been found to be between 91% - 95% and 99% - 100%, respectively, especially when using respiratory samples for both acid fast S^+C^+ and S^-C^+ samples [41]. However, interms of performance outcomes compared to culture, there seem to be no significant difference, but most importantly to note is the time differences in obtaining results for patient

management, as E-MTD takes one day (3 ½ hrs), while culture takes between 14-28 days to avail results [40].

Table 1.Comparison of commercially available (Amplicor, E-MTD) and Xpert MTB/RIF nucleic acid amplification tests for the detection of *M. tuberculosis* complex organisms.

Characteristic	Amplicor	E-MTD	Xpert MTB/RIF
Manufacturer	Roche	Gen-Probe	Cepheid
Amplification platform	PCR	TMA	Nested, real-time PCR
Target	16S rRNA	rRNA	rpoB gene
Ability to detect drug resistance	No	No	Yes (RIF)
Analytical sensitivity	≥ 20 orgs	N/A	131 CFU/ml
Clinical sensitivity	79- 92%	91– 95%	98.2 %
Clinical specificity	99 - 99.9%	98 – 100%	99.2%
Sensitivity for AFB smear-positive specimens	41- 73%	80 – 100%	73%
PPV	92.6 - 96.6%	83.3 – 100%	85%
NPV	98.6 - 98.7%	98.4 – 99.6%	91%
Control for amplification inhibitors	Yes	No	Yes
Prevention of carry-over contamination	Yes	No	Yes
Turn-around time after specimen decontamination	6.5 hrs	3.5 hrs	2 hrs
Instrumentation needed	Thermocycler, photometer	Heat block, luminometer	Xpert MTB/RIF instrument & cartridge
FDA-approved use	AFB smear-positive respiratory specimens	AFB smear-positive and –negative specimens	Co-infected pts with HIV, TB & MDR suspects

Definition of abbreviations: E-MTD; Enhanced *Mycobacterium tuberculosis* Direct Test, TMA; Transcription-mediated amplification, rRNA; ribosomal ribonucleic acid, PCR; polymerase chain reaction, CFU; colony forming units, RIF; rifampicin, AFB; acid fast bacilli, FDA; Food and drug administration, MDR; multi drug resistant.

1.8.3.2. NAATs in use today

There is a diagnostic delay and possible misdiagnosis in cases of EPTB, smear-negative TB and disseminated disease especially when HIV co-infection is involved , as smear microscopy solely will not be able to pick-up cases of TB in such situations [43].

1.8.3.2.1. What is the Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) assay?

This is a NAA platform that is automated, self regulatory, with limited hands on time, incorporating sample processing, nucleic acid extraction, amplification, TB detection and RIF-resistance [44]. This test avails results directly from raw samples through combining a short preparative process of the sample coupled with the automated PCR stage within two hours [45].

1.8.3.2.2. How Xpert MTB/RIF works?

The test can thus be carried out using unprocessed sputum samples or processed sputum pellets [46]. The only non-automated step for this assay involves the addition of a sample buffer to sputum, followed by transferring two mls of this sputum-buffer solution into the cartridge, where it then gets inserted into the Xpert chamber, availing results in approximately two hours, Figure 4. The test specifically targets MTBC organisms, while it allows for drug susceptibility testing (RIF-resistance) through amplifying the rpoB gene particularly the rifampin resistance determining region [46] which gets probed by molecular beacons for mutations within this RRDR [44].

1.8.3.2.3. Assay procedure for the Xpert MTB/RIF test

The lytic buffer is mixed in the ratio of 2 parts to 1 part of the sample (2:1 respectively), followed by shaking the mixture, which is then incubated at room temperature for at least 5 minutes followed by shaking once more. 2 ml of the sample reagent mixture is then transferred to the Xpert test cartridge and then placed on the Xpert chamber module. Proceeding steps thereafter become automated and the result is printable, with "MTB detected, RIF resistance not detected" [44].

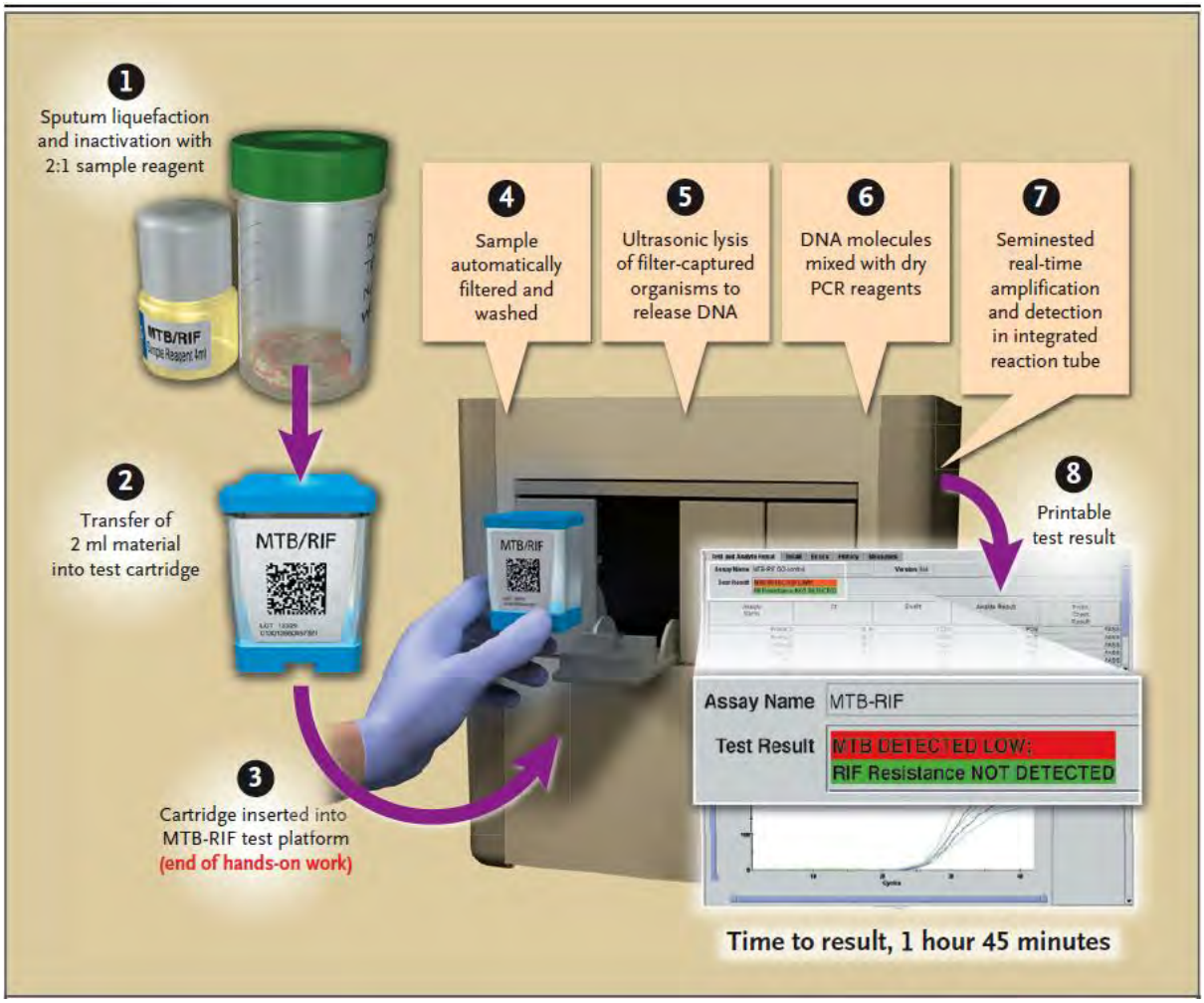


Figure 4: Xpert MTB/RIF test procedure [Adapted from Boehme *et al*. [44]].

1.8.3.2.4. Xpert MTB/RIF assay diagnostic accuracy and its impact

Xpert has a limit of detection (LOD) of at least 131 colony forming units (CFU) per ml of sputum, which thus is an index of the number of viable bacteria detected with 95% confidence [47].

As shown in Table 2, in a prospective, multicentre study, Boehme and colleagues noted that of the 561 patients tested amongst C^+ patients, 551 (98%) patients had S^+ TB, whereas, of the 171 patients with S^- disease, the test picked up 124 (73%) patients, while having a specificity of 99% (604 of 609 patients) [44]. In this study, out of 205 patients tested for drug resistance, 202 were picked up by the test (98%), while of the 514 patients with Rifampicin sensitive bacteria, the test correctly picked up 504 subjects (98%), where they concluded that was sensitive in the detection of TB, while also picking up RIF-resistance from unprocessed sputum, availing results within 2 hrs.

Helb *et al.* [47], in their study in Vietnam noted that out of 107 patients consecutively enrolled and suspected of TB, the test identified all 29 (100%) cases to be having C^+S^+ TB, while in S^- individuals, the test had a sensitivity of 72% (38 of 53, 95%CI 57-83%).

In another study, Theron *et al.,* noted that overall, Xpert MTB/RIF sensitivity was 95%, while having specificity of 94% amongst S^+ individuals, whereas in S^- individuals, the sensitivity was 55%, where the analysis was restricted to a ml of raw sputum [48].

Table 2. Summary of studies outlining the diagnostic accuracy of Xpert MTB/RIF in diagnosing TB.

Study	Country	Sample	Xpert Sensitivity	Xpert Specificity	Reference
Helb *et al* (2010)	Vietnam and Uganda	sputum	98 - 100	85 – 100	[47]
Blakemore *et al* (2010)	USA	sputum	100	100	[46]
Boehme *et al* (2010)	Peru, South Africa, India	Sputum	98	99	[44]

Theron *et al* (2011)	South Africa	sputum	95(88-98)	94(91-96)	[48]
Armand *et al* (2011)	France	Pleural fluid, bone	53(35-71)	n/a	[49]
Causse *et al* (2011)	Spain	Tissue biopsy, CSF, gastric aspirates	95(84-99)	100(99-100)	[50]
Friedrich *et al* (2011)	South Africa	Pleural fluid	25(9-49)	100(48-100)	[51]
Hillemann *et al* (2011)	Germany	Urine, gastric aspirate, tissue	77(61-87)	98(96-99)	[52]
Vadwai *et al* (2011)	India	Pus, body fluids, biopsy	81(76-85)	99(98-100)	[53]
Moure *et al* (2011)	Spain	Pleural fluid, abscess aspirate	58(49-68)	100(91-100)	[54]
Peter *et al* (2012)	South Africa	urine	40(22-61)	98(95-100)	[36]
Tortoli *et al* (2012)	Italy	Tissue biopsy, CSF, pleural fluid, urine, pericardial fluid, pus, gastric aspirates	81(76-86)	99(99-100)	[55]
Patel *et al* (2013)	South Africa	CSF	62(48-75)	95(87-99)	[56]
Theron *et al* (2013)	South Africa	BALF	93(77-98)	96(91-98)	[57]
Calligaro *et*	South	Tracheal	100	94	[58]

***al* (2015)**	Africa	aspirates

1.8.3.2.5. Limitations of the Xpert MTB/RIF assay

Amongst some of the Xpert assay drawbacks is that it is expensive to run a test (see Table 4), with the cost ranging from between USD22,63 to USD27,55 and USD17,000 per 4 module instrument, whereas a single sputum smear examination costs between USD1,13 to USD1,63 with LJ and MGIT cultures costing between USD13,56 to USD18,95 [59]. Another drawback is that the assay only tests for rifampicin resistance which is a platform that has too few occuring mutations. However, as a result of all these drawbacks especially the high costs involved and cold chain storage of buffers and cartridges, there is need for affordable, easy to operate and fast producing result systems bringing point of care to patients. Promising new diagnostic techniques, such as isothermal amplification platforms act as potential candidates in the diagnosis of TB.

1.8.3.3. Loop mediated isothermal amplification (LAMP)

Pathogen detection from clinical bio-specimens is paramount in directing appropriate therapy and treatment regimens in affected patients. Existing traditional techniques such as culture and microscopy tend to be time consuming amongst various factors thereby prolonging the time to diagnosis and treatment for the suffering patient.

The LAMP reaction is a novel test amplifying nucleic acids under isothermal conditions [37]. Unlike the polymerase chain reaction, the template strand need not be denatured [60]. It is a relatively simple technique, where a water bath is only needed to provide with the constant temperature, thereby making it a low cost technique which is favourable for use in high burdened, resource poor countries.

The loop mediated isothermal amplification assay relies on a DNA dependent polymerase that initiates amplification and elongates the template strand where four specially designed primers specifically recognize six distinct sequences on the target DNA [61, 62]. To further accelerate the reaction, two extra loop primers can be added to the reaction cocktail [63]. The LAMP technique has successfully been applied in the clinical diagnosis of HIV, HBV and SARS-CoV amongst other diseases.

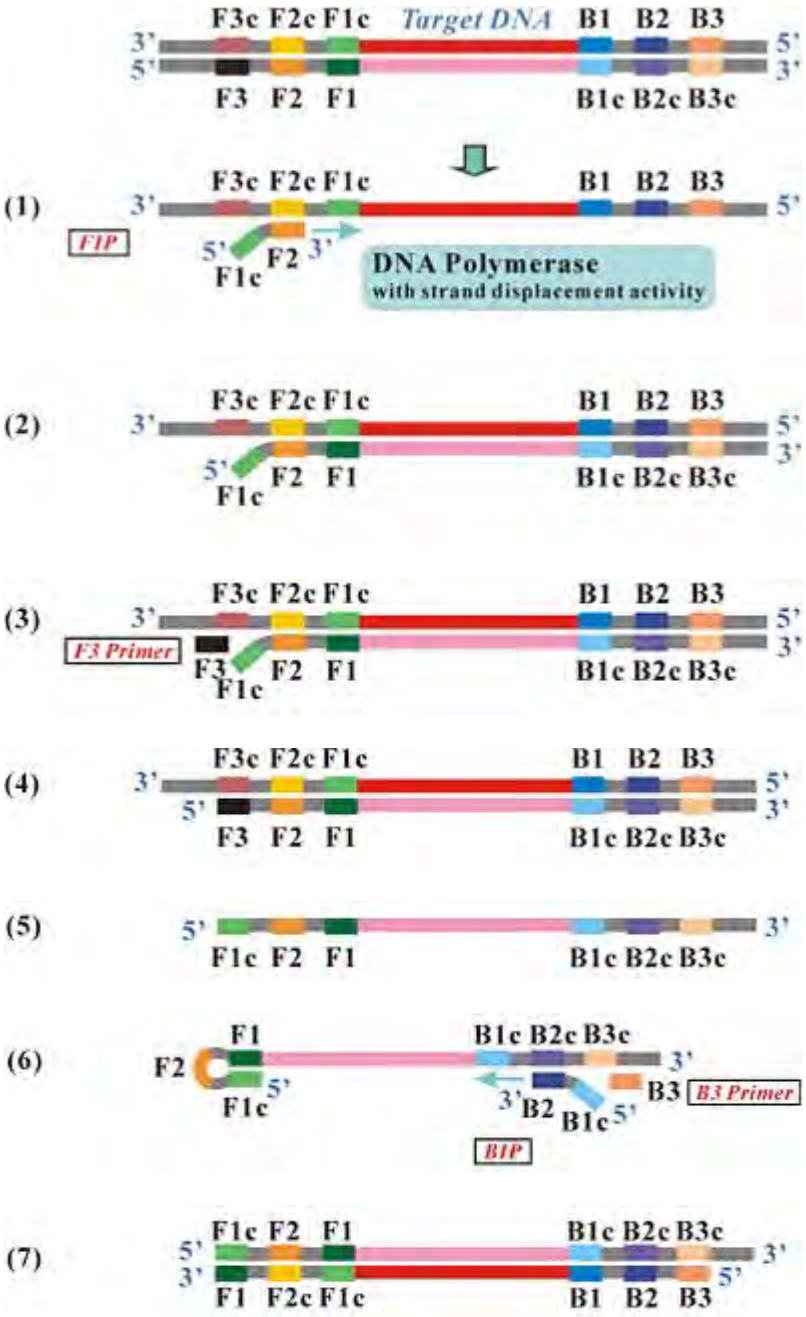
1.8.3.3.1. LAMP primer design

The test makes use of a pair of inner and another pair of outer primers, with the early stages of the reaction requiring all the two sets of the primers for efficient amplification. For the later stages of the reaction, only the inner primer pair is needed necessitating strand displacement synthesis. The outer primer pair is comprised of F3 and B3, while the inner primers are thus FIP and BIP. These inner primers do possess specific codes corresponding to the sense and antisense strands of the target mycobacterial nucleic acid where they are involved in priming activities [37]. The LAMP assay is thus a faster process due to additional loop primers [forward loop primer (LF) and backward loop primer (LB)]. A DNA polymerase enzyme catalyzes the reaction at a temperature range between 60-65 °C, which is also conducive for primer melting.

1.8.3.3.2. Molecular mechanism of the loop mediated isothermal amplification reaction

The LAMP start up cocktail possess a catalytic enzyme (Bst DNA polymerase), specific primers, dNTPs, and the target DNA template all carried out at a fixed isothermal temperature range for approximately an hour. Three sequential steps exist which are thus the synthesis of bio-molecules necessary to start up the reaction, cyclic amplification which is proceeded by the elongation of strands, and lastly the recycling phase of accumulating end products [37]. For the start up phase, a complex is formed between the forward inner primer (FIP) and F2c which is complimentary and found on the DNA of choice forming an FIP-F2c complex. Strand displacement DNA synthesis is initiated by a complex formed which is thus termed the F3-F3c complex, which results in the release of end products (loop structures with complementary sequences at one end). A stem loop structure of DNA is formed as a result of single stranded DNA (ssDNA) hybridizing with BIP and further priming by B3 signifying the cyclic phase of the reaction. The cyclic phase of the LAMP test involves the hybridization of the forward inner primer to the loop component of the stem creating a complex which through disruption of bonds leads to the formation of tandem arrays of gapped DNA with further inverted strands of the stem. The elongation and recycling

phases of the test, involve a further stem loop with complementary sequences to the original strand, and the gapped tandem repeat DNA that has been repaired by the enzyme which eventually serve as start up material for proceeding cyclic phases. The end product is a DNA molecule with several inverted stem loops of different sizes [37], see Figure 5.



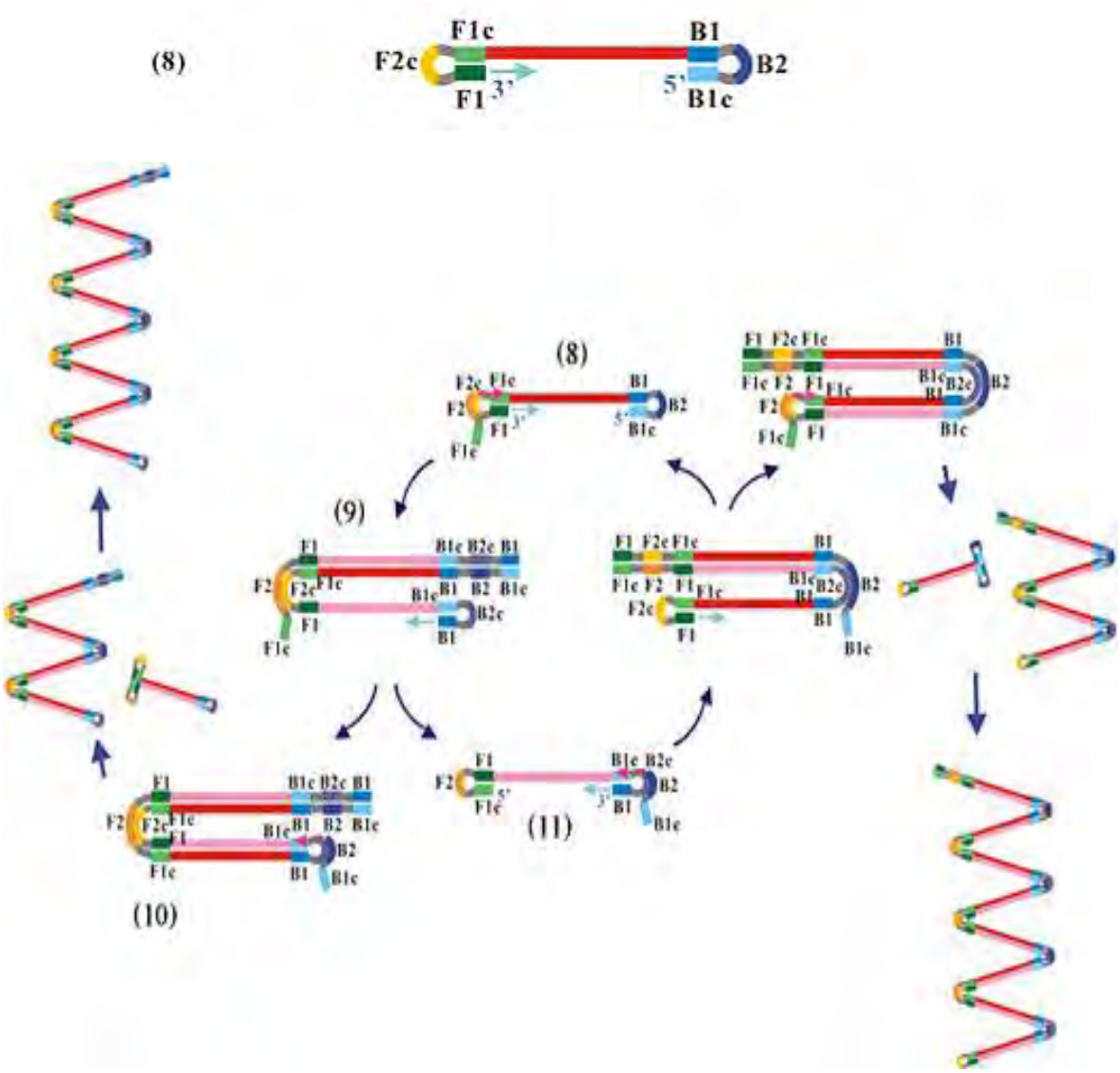


Figure 5: Molecular mechanism of the LAMP reaction [Adapted from Notomi *et al.* [37]].

1.8.3.3.3. Visualization of loop mediated isothermal amplification

Positive LAMP reactions can be visualized using a variety of methods. Gels can be used which are thus stained by dyes such as ethidium bromide and electrophoresis carried out to visualize products. The LAMP products visualized under UV light can be seen as stem and loop structures resembling a ladder-like appearance on the gel. The addition of a dye, SYBR Green 1 stain which has affinity to bind to DNA enables direct visualization [64]. In order to reduce contamination and allow for direct visualization, fluorescent detection reagent (FDR) is added to the reaction cocktail

before the initial amplification phase and viewed under UV light [60]. Real-time monitoring of turbidity through the use of a turbidimeter is also a way of monitoring positive test results, thus through the accumulation of salts (magnesium pyrophosphate) within the reaction mixture.

1.8.3.4. Loop mediated isothermal amplification platforms targeting different MTBC genes

1.8.3.4.1. LAMP targeting the gyrB gene

a. Iwamoto and colleagues in their study specifically targeted the gyrB gene to detect mycobacterial species in the sputum of suspected TB patients [62]. In this study, they had a cohort of 24 MTBC species and 7 non-mycobacterial species, where they held their reaction mixture at a fixed temperature of 63 °C [62].

b. Boehme *et al.*[65], carried out a multicentre trial in Peru, Tanzania, and Bangladesh using processed sputum and the reaction mixture was maintained at 67°C for 40 min. In this study, they changed parameters such as temperature and number of primers while focusing at the gyrB gene. Their study findings were that, LAMP had a sensitivity of 97% (173 of 177, 95% CI 96-99%) amongst S^+C^+ samples, while in S^-C^+ samples, the sensitivity was 48% (21 of 43, 95% CI 34-64%).

1.8.3.4.2. LAMP targeting the rrs gene

In a study in Nepal, Pandey *et al.*[66], designed six species specific primers targeting the 16S rRNA gene found in MTBC organisms, where they analyzed 200 sputa samples with an isothermal temperature maintained at 64 °C for an hour. MTBC species exhibited efficient DNA amplification with this system where in C samples, the sensitivity was of the test was 100% (96/96) [66].

1.8.3.4.3. LAMP targeting the Insertion Sequence 6110 (IS6110) gene

In a study by Aryan *et al.* [33], the repetitive IS6110 sequence was used as a target gene, as this gene exists conservatively within the MTBC species. They established

that their assay was almost 20 times highly sensitive than the basic conventional PCR for the same gene. Also in comparing their findings where they targeted the IS6110 to the previously mentioned genes (gyrB and rrs), sensitivity was 50: 20 times higher respectively.

1.8.3.5. Advantages of LAMP

LAMP has the ability to amplify DNA at low constant isothermal temperatures allowing low cost equipment to be used for its operation [33, 65, 67]. The test's cocktail incorporates primers, the DNA dependent polymerase Bst, and the sample which are maintained at specific temperatures usually between 60-65°C and upon amplification, results visualized and this is done in a single step process [37]. Conventional PCR involves the initial denaturation of the template whereas the LAMP test is void of that stage. There is no need for a thermal cycler for LAMP to proceed making it a user friendly and simple diagnostic technique in clinical medicine especially in less developed countries with limited resources [68]. The constant isothermal temperatures coupled with the use of a single catalytic and proof reading polymerase makes the loop mediated isothermal amplification assay a quicker technique [33, 37, 62, 66]. LAMP-positive results are easily visualized as a white precipitate due to the accumulation of magnesium pyrophosphate ions within the reaction mixture making it turbid [69]. Unlike conventional PCR, the LAMP test is tolerant to inhibition greatly especially from items like culture medium and salts that build-up during the process which can have a direct bearing effect on the amplification efficiency especially during PCR [68]. Comparing the LAMP assay interms of end products, to conventional PCR, it has approximately 50 times more efficiency [33].

Techniques that alleviate and result in more rapid diagnoses, benefit not only the patient interms of early treatment but can also ease pressure on the possibly dwindling resources by governments and should be rolled-out within high burdened, resource limited countries. It is however for these factors that the LAMP test with attributes that may enable its application in less privileged set-ups has been developed and thus could possibly offer point of care molecular testing within such settings.

Table 3.Commercially available nucleic acid amplification tests (NAATs) for tuberculosis diagnosis in summary.

NAAT	Method/Principle	Manufacturer
Amplified *M. tuberculosis* Direct® Test (AMDT)	Transcription-mediated amplification of rRNA transcripts. Product detection is performed via chemiluminescence	Gen-Probe Inc. San Diego, California
Roche Amplicor® MTB	PCR amplification of 16s rRNA. Product detection is performed via colorimetric detection	Roche Molecular Systems Branchburg, New Jersey
Cobas® Amplicor	PCR amplification of 16s rRNA	Roche Molecular Systems Mannheim, Germany
BD-ProbeTec Direct And the BD-ProbeTec ET	Strand Displacement Amplification (SDA) technology for the direct, qualitative detection of 16s rRNA and the *M. tuberculosis* insertion sequence IS6110	Becton-Dickinson Diagnostic Systems Sparks, Maryland
Xpert® MTB/RIF	Semi-quantitative nested real-time PCR in-vitro diagnostic test for the detection of M. tuberculosis complex DNA, and rifampicin-resistance associated mutations of the rpoB gene	Cepheid, Sunnyvale, California
Loop-mediated Isothermal Amplification (LAMP)	Isothermal amplification and visual read-out with ultra violet fluorescence	Eiken Chemical Co. Ltd., Japan

1.8.3.6. Other amplification platforms

1.8.3.6.1. Nucleic acid sequence based amplification (NASBA; bioMérieux, Boxtel, The Netherlands)

Nucleic acid sequence-based amplification is a commercially available isothermal, *in vitro* amplification procedure that uses RNA as the specific amplification target [70, 71]. The assay employs the simultaneous activities of reverse transcriptase, RNase and RNA polymerase enzymes which result in a single stranded-RNA product which is antisense to the original RNA template [37, 72]. NASBA has been successfully applied as a rapid system in the detection of astroviruses and in the monitoring of cytomegalovirus infections in transplant recipients as well.

It makes use of the simultaneous enzymatic activities of avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H, and T7 RNA polymerase, under isothermal conditions (Figure 6). The constant temperature maintained throughout the amplification reaction allows each step of the reaction to proceed as soon as an amplification intermediate becomes available. Products of NASBA are single stranded and, thus, can be applied to detection formats using probe hybridization [70].The end products of NASBA can be detected using gel electrophoresis, fluorescence probes (real-time NASBA), and colorimetric assay [73, 74]. The FDA has approved the technique in NucliSence formulation (NASBAECL) for molecular detection of some microorganisms such as HCV and HIV-1 [74]. NASBA is highly compromised in its effective use as a diagnostic technique in that it requires relatively low temperatures of about 40 °C for amplification [37].

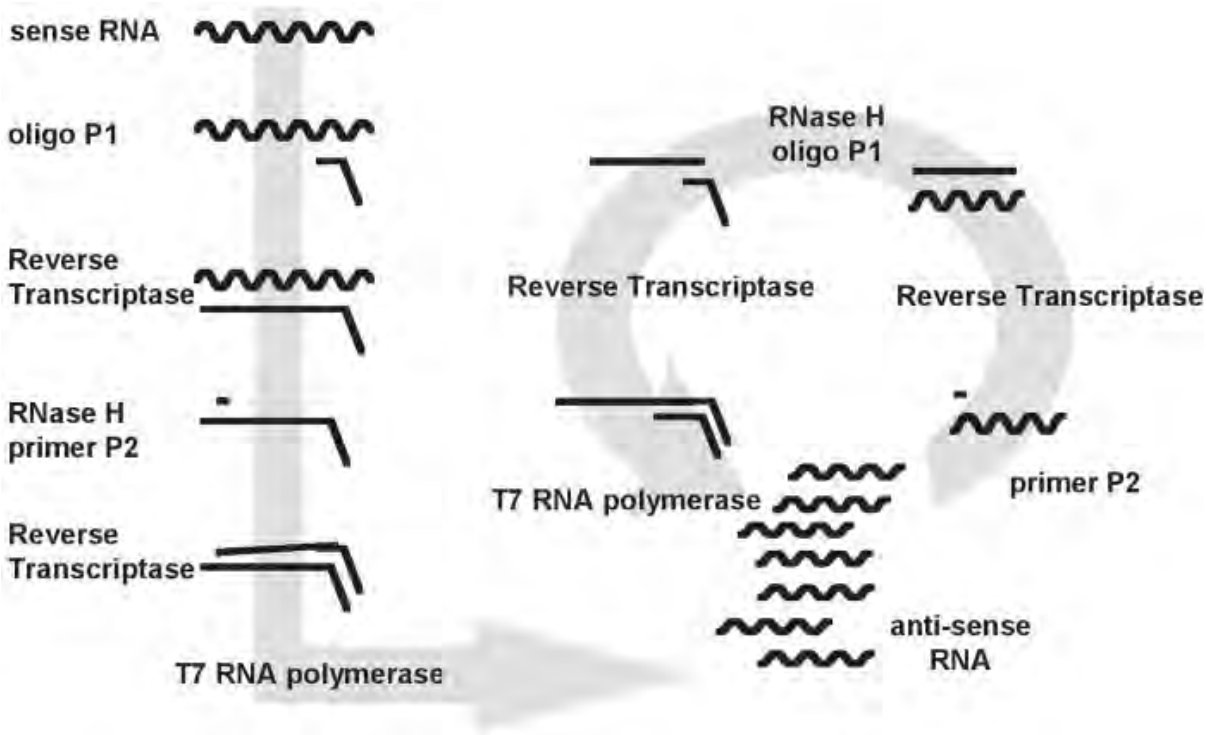


Figure 6: Simplified diagram representing NASBA [Adapted from Notomi *et al*. [37]].

The straight arrow represents the initiation phase while the circular arrow represents the cyclic phase. The activities of reverse transcriptase, RNase H, T7 RNA polymerase, and the primer-binding activities are also indicated.

1.8.3.6.2. Transcription mediated amplification

Reverse transcriptase is used to copy rRNA into a transcription complex which is then transcribed by DNA-directed RNA Polymerase to produce more rRNA molecules [40]. These transcripts serve as templates for reverse transcription and further amplification. The specificity of the assay primarily depends on the specificity of the oligonucleotides used in the hybridization assay. A TMA-Based Kit for the detection of MTB in sputum specimens was developed thus the MTD-Test, Gen-Probe, San Diego and the procedure's turnaround time is 3-4 hrs of obtaining a processed specimen.

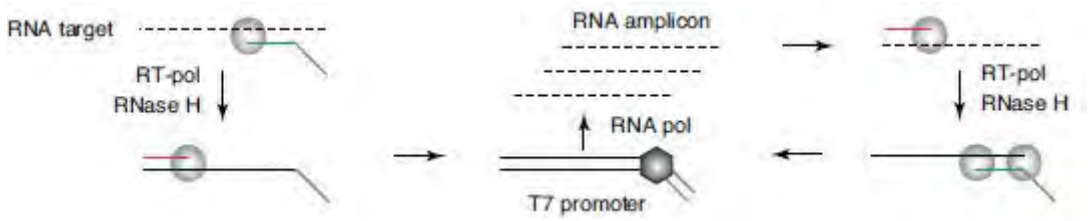


Figure 7: Simplified diagram of the TMA method [Adapted from Niemz *et al*. [75]].

The RNA target is converted to ds cDNA with a promoter region through reverse transcription, followed by RNase H degradation of the original strand. DNA polymerization is initiated by a second primer. RNA polymerase (pol) amplification creates products that feed back into the original reaction [75].

1.8.3.6.3. Strand displacement amplification (SDA)

This is an isothermal, *in vitro* method of amplifying a DNA target up to 10^8 fold at 40 °C [32]. Such a technique is based upon the ability of a restriction enzyme to nick a hemi modified recognition site allowing the polymerase to displace a downstream DNA strand during replication [32, 37]. SDA has been used to amplify only a single target species in each reaction mixture, Figure 8.

Recent advances have allowed the extension of the capability of such a technique to allow simultaneous and efficient amplification of two target sequences coupled with an internal control molecule designed to act as a standard (Multiplex SDA technique) which has the advantages of allowing the use of a single pair of amplification primers on a pair of different targets, thus ensuring that amplification proceeds at comparable rates and minimizing background amplification reactions as a result of errant priming events. The multiplex SDA method allows the IS6110 sequence to be amplified providing amplification level specificity for the MTBC strains while the other target being the 16S ribosomal gene common to all clinically relevant mycobacterial species allowing mycobacterial genus specificity at the level of amplification.

SDA has the disadvantages of having increased backgrounds due to digestion of irrelevant DNA contained in the sample, while it also requires the use of costly modified nucleotides as a substrate making it impossible to be implemented in the resource disadvantaged country settings as a reliable diagnostic technique [37].

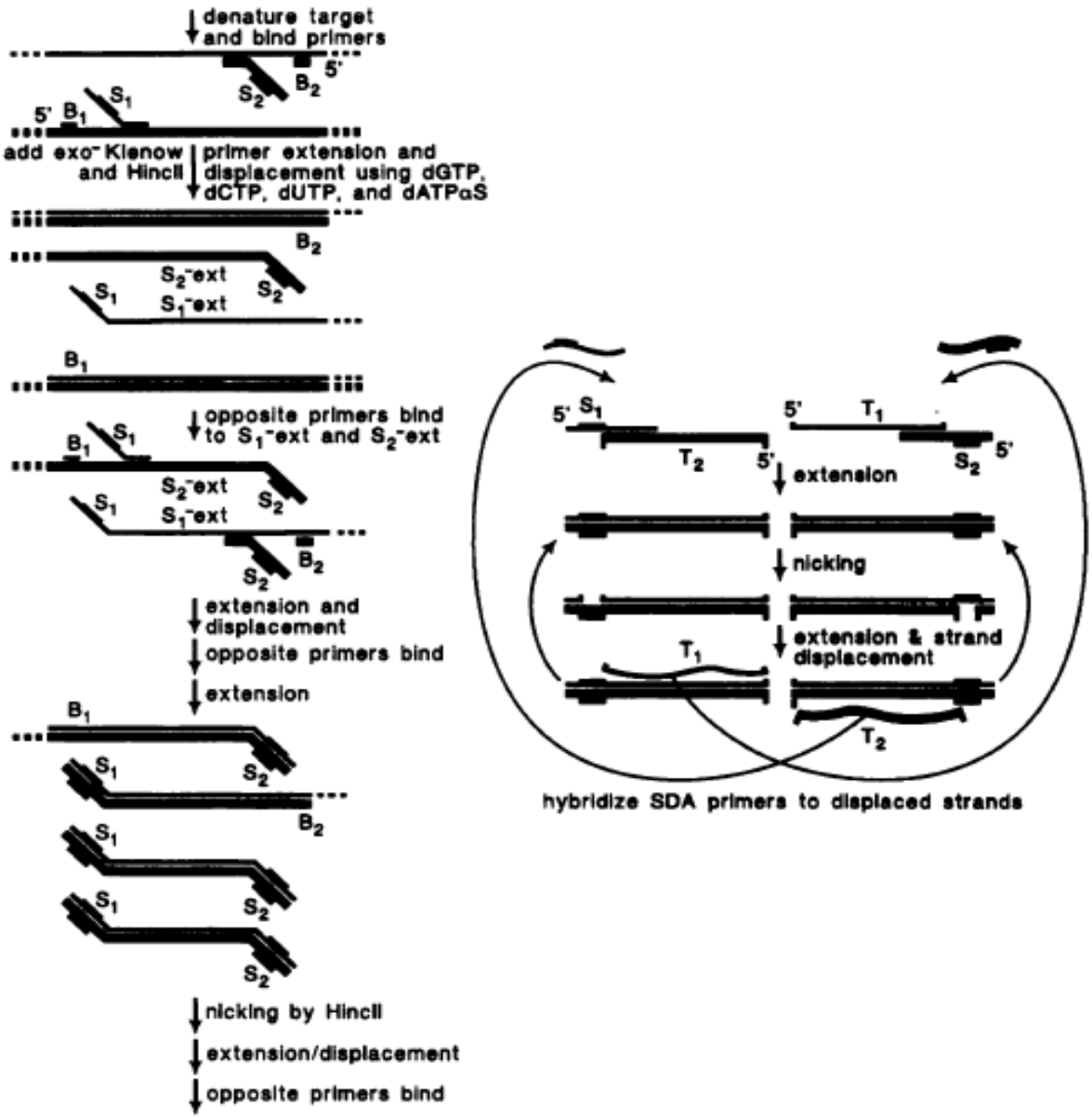


Figure 8: Simplified diagrammatic representation of single-target SDA [Adapted from [76]].

The target DNA is heat denatured in the presence of all reagents except the restriction enzyme and polymerase, after which amplification proceeds at approximately 40 °C after the cooling step and addition of the enzymes (restriction enzyme and polymerase) [74].

Table 4. Comparison of the sensitivity, drug susceptibility testing, cost and the time to diagnosis of different diagnostic tools for active TB in children and HIV-TB co-infected patients.

Test	Sensitivity (%)				Drug sensitivity	Cost in USD/test	Time to diagnosis	WHO endorsement
	Children	HIV-infected	HIV-uninfected	Smear-negative patients				
POC LAM	n/a	50	20	n/a	No	3.5	25 mins	No
LAM ELISA	n/a	50	20	n/a	No	3.5	1-2 days	No
Xpert MTB/RIF	75	82	90	70	Yes	14	2 hrs	Yes
Florescence smear microscopy	20	40	70	n/a	No	3.5	4 hrs - 2 days	Yes
Roche Amplicor	66	overall 85		66	No	15	4 hrs - 2 days	No
MTBDRplus	overall sensitivity is 98% in S+ pts			60	Yes	15	4 hrs - 2 days	Yes
MODS	40	90	95	n/a	Yes	2.5	7 - 14 days	Yes
MGIT	40	80	90	n/a	Yes	16	10 - 14 days	Yes
LAMP	overall sensitivity is 84%			60	No	6	2 hrs	No

Definition of abbreviations. Only tests commercially available have been included which are found in South Africa. GeneXpert MTB/RIF assay for frontline TB diagnosis in TB clinics. Line Probe Assays (LPA); genotypic drug susceptibility testing using Genotype MTBDRplus (Hain lifesciences, Nuhren, Germany), DST; phenotypic drug susceptibility testing using automated liquid culture; Mycobacterial-Growth-In-Tube (MGIT, Becton Dickinson Diagnostics, USA); LAMP (Eiken, Tokyo, Japan), TB LAM ELISA (Alere,

Waltham, MA, USA), MODS; microscopic observed drugs susceptibility; TBMODS Kit® (Hardy Diagnostics, Santa Maria, CA, USA), Cobas Taqman MTB (Roche Molecular Diagnostics, Pleasanton, CA, USA). S+; smear positive, N/A; not available [77, 78]

2. CHAPTER 2

Diagnostic accuracy of the loop mediated isothermal amplification (LAMP) assay for the detection of *Mycobacterium tuberculosis* on sputum.

ABSTRACT

Background

The accuracy of nucleic acid tests such as LAMP when performed on sputum of patients suspected of pulmonary TB is limited. This study evaluated the diagnostic accuracy of the promising novel isothermal LAMP assay on sputum collected from South African patients suspected of TB.

Methods

301 South African patients had their sputa collected. Two spot sputa, approximately 4 ml were collected per patient, with 60 µl of sputum being used for the LAMP test. Liquid culture was performed on sputa collected at the same time as that used for the LAMP test, where liquid culture served as a reference standard for definite TB. We evaluated accuracy according to HIV and smear microscopy status, when these data were available. Also, the relationship between test performance and TTP was compared.

Results

Of the 297 patients included, 58 were culture confirmed cases for TB (31% were HIV-positive). The sensitivity of LAMP [77% (95% CI 67- 84%] was similar to that of smear microscopy [67% (56-76%); p=0.134] while the specificity of LAMP was different to that of smear microscopy [91% (86-94%) vs. 99.5% (97-99%); p<0.003, respectively]. The sensitivity of LAMP was similar in HIV-infected vs. –uninfected patients (70% vs. 85%; p=0.545, respectively). Amongst the 29 smear–negative patients, the LAMP test picked up 14 patients to be positive. LAMP-positivity had a

significant shorter time to culture-positivity; [16 days (4-50)] than LAMP-negative patients; [40 days (6-50), **p<0.0001]**.

Conclusion

The LAMP assay had modest sensitivity, while it also had incremental yield over smear microscopy. The test also had suboptimal accuracy for the detection of pulmonary TB as the false positive rate was too high. Further development maybe required on the stability and sensitivity of the LAMP test for its application in the clinical diagnosis of pulmonary TB.

2.1. INTRODUCTION

Traditionally the diagnosis of TB disease has been done through culturing bacteria on agar plates followed by determining the phenotypic and serological properties of the pathogen [79], [80]. Attempts have been made using biochemical tests, however such techniques have insufficient sensitivity to detect low levels of pathogen and are often dependent of first culturing the pathogen [81]. Molecular techniques such as the polymerase chain reaction (PCR) can be used to speed up diagnosis and increase sensitivity and specificity of pathogen detection.

Despite the availability of numerous diagnostic methods, there is no single rapid, sensitive, inexpensive and non- laborious method for field diagnosis of diseases such as TB. PCR techniques have significantly increased our ability to detect TB infection, but its requirement of a high-precision thermal cycler has prevented it from being widely used in the field or by private clinics as a routine diagnostic tool. The recently endorsed Xpert MTB/RIF assay recommended by the WHO as a "first-line" diagnostic test in endemic countries and for management of multi-drug resistant TB cases serves and incorporates most of these POC attributes and is useful for use in the field in TB diagnosis [82, 83].

Alternatively, isothermal nucleic acid amplification methods, which require only a simple heating block and obviate the need for thermal cycling, have been developed to offer feasible platforms for rapid and sensitive detection of a target nucleic acid. These include nucleic acid-based amplification (NASBA), loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA) and ramification amplification [37, 72, 84, 85].

Boyle *et al.* [84], evaluated the RPA assay performance which is a rapid, low temperature isothermal DNA amplification reaction against indirect smear microscopy and found that it had a sensitivity of 91% (95% CI 85 - 98%) and specificity of 100% compared to smear microscopy which had 86%.

The loop-mediated isothermal amplification (LAMP) assay is a gene amplification procedure, in which the reaction proceeds at a constant temperature catalyzed by one type of enzyme. It is a rapid assay (approximately between 35 to 60 mins [62]) and possess simple features making it different from the existing genetic tests currently available [37]. The LAMP method is able to amplify between 5 to 50 copies of DNA in less than an hour with no special reagents required [86]. The procedure is characterized by the use of 4–6 different primers specifically designed to recognize between 6-8 distinct regions on the target gene relying on autocyclic strand displacement DNA synthesis catalysed by the *Bst* DNA polymerase large fragment, which has high specificity and sensitivity under isothermal conditions [62] making it to be a highly specific assay since it allows MTBC detection [33]. The reaction process proceeds at a constant temperature (60–65 °C) [37], [63]. Furthermore, in a LAMP assay, all steps from amplification to detection are conducted within one reaction tube under isothermal conditions. Visual detection is enhanced as a result of high amounts of insoluble magnesium pyrophosphate produced and fluorescence allowing it to be adopted in resource poor countries [33].

The above advantages can be used to prevent contamination, which can occur in PCR during the transfer of samples containing amplicons from tubes to gels for electrophoretic confirmation and thus omit the need for complicated temperature control, as required for PCR [60]. Owing to these advantages, the LAMP assay does not require well-equipped laboratories to be performed, with the procedure being easily standardized among different laboratory set ups.

LAMP based platforms have been developed targeting the gyrB gene [62, 65], the rrs gene [66] and the IS6110 [33] useful in TB detection. This technique has also been successfully applied to the detection of infectious agents such as viruses, parasites and fungi.

2.2. HYPOTHESIS, AIMS AND OBJECTIVES

2.2.1. Hypothesis

The LAMP assay is an accurate TB diagnostic tool which will improve the diagnosis of TB.

2.2.2. Aims

To determine the performance outcomes of the manual LAMP platform on single spot sputum collected from TB suspects at primary health care centres in Cape Town, South Africa vs. concentrated fluorescence smear microscopy and liquid culture for the diagnosis of pulmonary TB.

2.2.3. Objectives

- To evaluate the sensitivity and specificity of LAMP for the detection of TB vs. liquid culture as a reference standard.
- To compare the diagnostic accuracy of LAMP in HIV-positive and -negative individuals.

2.3. MATERIALS AND METHODS

2.3.1. Study design

This project was embedded in a bigger multi-centre, blinded, cross-sectional study to determine the performance of LAMP in patients with symptoms of pulmonary TB (PTB) in comparison to conventional methodologies (concentrated smear microscopy and liquid culture). However, for this particular analysis only the Cape Town site specific data was used.

2.3.2. Study flow

TB suspects provided two sputum specimens each. A direct smear and a direct LAMP test were performed from each sputum sample. After NALC-NaOH decontamination, a concentrated smear, an LJ culture, and a MGIT culture were performed from each sputum sample, Figure 9. The second sputum also underwent conventional DNA extraction (Qiagen) from which a LAMP test was performed. The first positive culture from each patient underwent confirmation of *M. tuberculosis* species by MPT64 antigen detection.

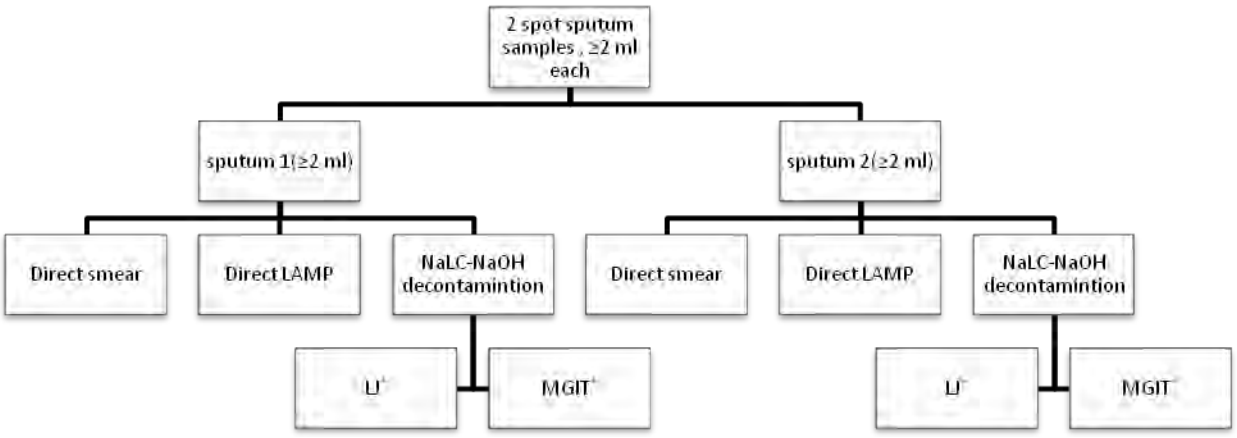


Figure 9: Sample flow and diagnostic tests performed at enrolment on each sputum specimen.

Definition of abbreviations. LJ=Lowenstein Jensen, MGIT=Mycobacterial growth indicator tube, NALC-NaOH= N-acetyl-L-cystein-NaOH. * Confirmation of MTB in all positive cultures with Capilia rapid test or conventional methods. 87 patients were culture-positive, 59 patients were smear-positive and 85 were LAMP-positive.

2.3.3. Sample size

In total, 301 patients with suspicion of PTB were enrolled.

2.3.4. Inclusion and exclusion criteria

2.3.4.1. Inclusion criteria

- Persistent cough (≥2 weeks) and at least one other typical symptom of PTB (fever, night sweats, malaise, recent weight loss, contact with active case, hemoptysis, chest pain, loss of appetite)
- Provision of written and signed informed consent.
- Patient aged 18 years or above.
- Patients able to provide two expectorated sputum specimens (2 sputum specimens of ≥1.5 ml each) over the course of 2 days.

2.3.4.2. Exclusion criteria

- Patients receiving any anti-TB medication, including fluoroquinolone and aminoglycosides in the 60 days prior to testing
- Patients with only extra-pulmonary disease
- Inability to provide informed consent (e.g. mentally impaired).

2.3.5. Subject recruitment

Patients who had symptoms consistent with PTB presenting to health facilities were asked to participate. Patients were identified by regular clinic staff or study personnel during an initial interview. Patients would be told that participation is voluntary and that they have the opportunity to ask questions individually. A consent form would be

signed by all participating patients. Eligible patients who signed the informed consent form will have their medical history taken, and they will undergo a clinical examination, a chest X-ray, HIV testing (only if part of the routine standard of care), and they will be asked to provide two sputum samples (see Figure 9). Samples had to be collected before the patient starts TB treatment. All results were recorded in the Case Report Form (CRF). Study participants with incomplete CRFs were withdrawn and a new study subject enrolled instead. Statistical analysis was done using a statistical software; OpenEpi (Open Source Epidemiologic Statistics for Public Health, Version 2.3. www.OpenEpi.com) and Graphpad Prism (version 5).

2.3.6. Laboratory based methods

2.3.6.1. Sputum sample Preparation (10 - 20 min)

60 µL of sputum was transferred from a sputum cup to a heating tube, using an Eiken disposable micropipette. The heating tube was inverted 3-4 times and placed in the heating block at 90°C for 5 min to lyse and inactivate the mycobacteria. The heating tube was removed from the heating block and allowed to cool down for 2 minutes and immediately after that attached to an absorbent tube and contents shaken until all the powder had dissolved. An injection cap was then attached on the heating tube and tightly screwed to pierce the seal, **Figure 10.**

2.3.6.2. Reaction Tube Preparation (5-15 min)

The heating tube's nozzle was inserted into a reaction tube and 30-35 µl of DNA solution was transferred. The reaction tubes were flicked down to collect the DNA solution in the cap and allowed to stand for 2 min before inverting the tubes upright again to collect the solution at the bottom of the tube.

2.3.6.3. Amplification (45 min)

The incubators temperature was set at 67°C.Reaction tubes were loaded into the heating block and amplification started through switching on the machine. Amplification was stopped automatically after a 5 min inactivation step.

2.3.6.4. Visual readout

The reaction tubes were placed in the UV reader integrated into the heating block and the results were recorded. Reaction tubes were stored without opening the vials to minimize contamination from amplicons, **Figure 10**.

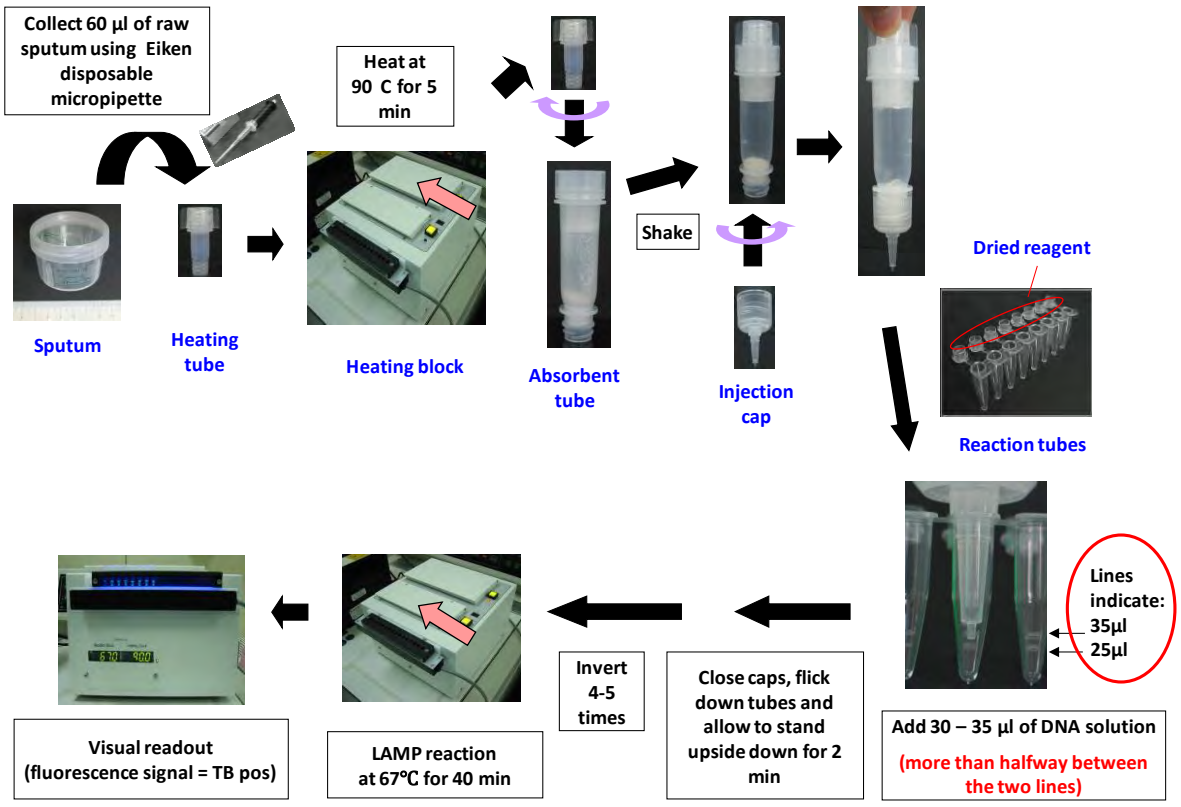


Figure 10: TB Loopamp™ procedure showing the consumables, tubes and standard operating procedure [87].

2.3.6.5. Definitions applied for the analysis

The diagnosis of individuals was based on the detailed characterization criterion done by a clinician blinded to the assay results. **Definite TB**; a clinical presentation compatible with TB having at least one spot sputum sample culture-positive for *M. tuberculosis* (culture-positive TB), **Non-TB**; no bacteriologic evidence of TB based on smear microscopy and culture and when available no radiologic evidence to support the diagnosis of TB and **Indeterminate TB**; where either the culture or chest x-ray results (or both) were unavailable, and or the patient was lost to follow-up or transferred to another centre, making it impossible to confidently rule-out or rule-in TB. These patients were excluded from the analysis.

2.4. RESULTS

Table 5.Demographic information and clinical characteristics stratified by smear status

Demographic/clinical characteristics	Study cohort	Smear-positive, culture-positive	Smear-negative, culture-positive	p-Value
# of TB suspects	**297**	**58**	**29**	
Median Age (IQR)	35 (28-44.3)	35 (28-44.1)	35 (28-44.2)	0.114
Males	179 (60)	40 (69)	17 (59)	0.352
Females	118 (40)	18 (31)	12 (41)	0.352
HIV-positive patients[+]	108 (36)	18 (31)	19 (66)	0.003
Median CD4 count (cells/ml) in HIV-positive patients (IQR)[≠]	257.5 (168-381)	255.5 (145.5-381)	260.5 (145.5-381)	0.542
Chest X ray compatible TB likely patients	95 (32)	50 (86)	16 (55)	0.002

Definitions of abbreviations: TB; tuberculosis

*** P value comparisons are for smear-positive, culture-positive vs. Smear-negative, culture-positive groupings.**

[+] Excludes 39 patients who refused HIV testing and or with no data recorded.

≠ Excludes 18 patients who were HIV-positive but with no CD4 count data.

Figure 11 depicts how patients were categorised and placed into different diagnostic sub-groupings. 297 patients with suspected TB were eligible for inclusion into the analysis (Figure 11). The patient demographic and clinical characteristics are shown in Table 5. A total 87 (29 %) of the 297 patients had definite TB. Of these, 58 (67%) of 87 patients had smear-positive, culture-positive TB, whereas 29 (33%) of 87 had

smear-negative, culture-positive TB, Table 5. A total 60 (20 %) patients were classified as probable TB, whereas 150 (50%) patients were classified as non-TB.

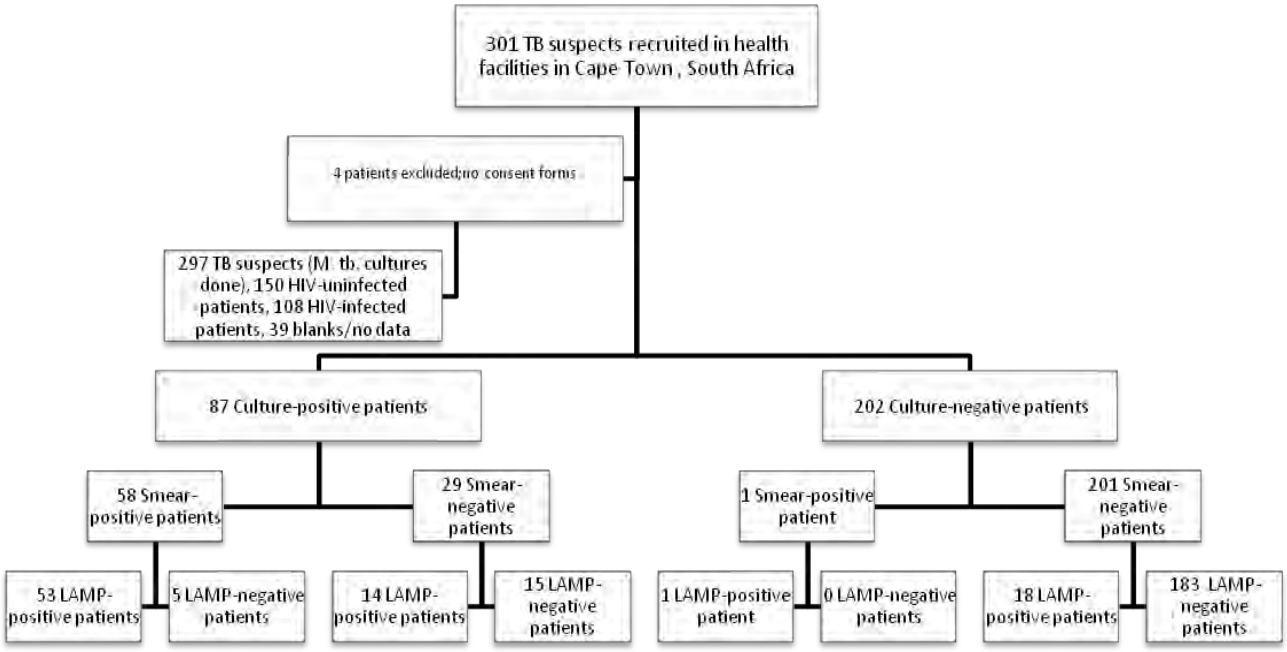


Figure 11: Flow diagram outlining patient enrolment, diagnostic category including HIV status in patients within primary health care facilities in Cape Town, South Africa used for the analysis.

2.4.1. Diagnostic accuracy of LAMP on sputum

Of the 87 culture-positive patients, 67 had LAMP-positive sputum. The sensitivity of LAMP was 77% (67 - 84%) which was similar to smear microscopy performed on the

same sputum, which had a sensitivity of [67% (56 - 76%); p=0.134; Table 6]. 18 of the 202 culture-negative patients had LAMP-positive sputa, resulting in a specificity of 91% (86 - 94%) which was significantly different to smear microscopy [99% (97 - 99%); p<0.003]. The positive predictive value (PPV) of LAMP was 79% (69 - 86%; 67/85) which was significantly different to smear microscopy which had a PPV of [98% (91-99%; 58/59; p<0.003; Table 6], while there was no difference in the NPV of the LAMP test and smear microscopy [(90% vs. 87%); p=0.363; respectively, Table 6].

2.4.2. Effect of HIV co-infection on LAMP performance

HIV co-infection did not impact on the sensitivity and specificity of LAMP. The LAMP test sensitivity in HIV-infected patients [70% (54 - 82%); p=0.162] was similar in its performance in HIV-uninfected patients, 85% (69 - 93%), while also having similar specificity [91% (81 - 96%); in HIV-infected patients] compared to a specificity of [89% (82 - 93%); p=0.702, in HIV-uninfected patients]. The specificity of LAMP in HIV-uninfected patients was 89% (82 - 93%) which was different to smear microscopy performed on the same sputa, [100% (96 - 100%); p=0.0001], while also the NPV of LAMP was not the same as that of concentrated smear microscopy [85% (76 - 91%) vs. 95% (89 - 98%); p=0.001; respectively, Table 6].

2.4.3. Accuracy of the LAMP assay in smear-negative individuals

The sensitivity of the LAMP test in smear-negative individuals was 40% (23- 59%) which was significantly different from LAMP performed on the same sputum, which had a sensitivity of [77% (67 - 84%); p<0.0000001, Table 6]. HIV co-infection didn't impact on the sensitivity and specificity of the LAMP assay in smear-negative cases, whereas it had a significant influence in NPV between HIV-infected and -uninfected patients (87% vs. 100%, p=0.0002), respectively.

Table 6.Diagnostic accuracy of LAMP performed on spot sputa and stratified by HIV status. (Liquid culture positivity for *Mycobacterium tuberculosis* serves as a reference standard).

	All patients (n=297)		**HIV-uninfected (n=150)***		**HIV-infected (n=108)***	
	sens. (95% CI) n/N	**spec.** (95% CI)¥ n/N	**sens.** (95% CI) n/N	**spec.** (95% CI)¥ n/N	**sens.** (95% CI) n/N	**spec.** (95% CI)¥ n/N
Conc. Smear microscopy (%)	**67** (56-76) 58/87	**99.5** (97-99) 201/202	**79** (62-89) 26/33	**100** (96-100) 112/112	**49** (33-64.1) 18/37 **(P=0.011)**+	**99** (92-99) 67/68 (p=0.378)+
LAMP (%)	**77** (67-84) 67/87 (P=0.134)ł	**91** (86-94) 184/202 **(p<0.003)**ł	**85** (69-93) 28/33 (P=0.545)ł	**89** (82-93) 100/112 **(p=0.0001)**ł	**70** (54-82) 26/37 (p=0.162)+ (p=0.065)ł	**91** (82-96) 62/68 (p=0.702)+ (P=0.064)ł
LAMP performed in smear-negative individuals (%)	**40** (23-59) 10/25 **(p<0.0000001)**≠	**91** (86-94) 183/201 (p=0.988)≠	**0** 0 0	**89** (82-94) 100/112 (p=0.999)≠	**44** (23-67) 7/16 (p=0.082)≠	**91** (82-96) 61/67 (p=0.725)+ (p=0.98)≠
	PPV (95% CI) n/N	**NPV** (95% CI) n/N	**PPV** (95% CI) n/N	**NPV** (95% CI) n/N	**PPV** (95% CI) n/N	**NPV** (95% CI) n/N
Conc. Smear microscopy (%)	**98** (91-99) 58/59	**87** (82-91) 201/230	**100** (87-100) 26/26	**94** (88-97) 112/119	**95** (75-99) 18/19 (p=0.422)+	**78** (68-85) 67/86 **(p=0.0008)**+
LAMP (%)	**79** (69-86) 67/85 **(p=0.0003)**ł	**90** (85-93) 184/204 (p=0.363)ł	**70** (55-82) 28/40 **(p=0.001)**ł	**95** (89-98) 100/105 (p=0.727)ł	**81** (65-91) 26/32 (p=0.291)+ (p=0.207)ł	**85** (76-91) 62/73 **(p=0.023)**+ (P=0.269)ł
LAMP performed in smear-negative individuals (%)	**36** (21-54)	**92** (88-95)	**0** 0	**100** (96-100)	**54** (29-77)	**87** (77-93)

	(p=0.00005)$^{\neq}$ 10/28	183/198 (p=0.436)$^{\neq}$	0	100/100 **(p=0.034)**$^{\neq}$	7/13 (p=0.082)$^{\neq}$	61/70 **(p=0.0002)**$^{+}$ (p=0.713)$^{\neq}$

Definition of abbreviations: CI= confidence interval; NPV= negative predictive value; PPV= positive predictive value.
***Excludes 39 patients who refused testing and or with no data.**
¥ Specificity calculations were based on culture-negative samples obtained from culture- negative group (non-TB).
$^{+}$P-values marked with a $^{+}$ are for comparisons according to HIV-status.
† P-values marked with a † are for comparisons between LAMP and smear microscopy
‡ P-values marked with a ‡ are for comparisons between LAMP and LAMP performed in smear-negative patients

2.4.4. Diagnostic accuracy of LAMP

Overall, LAMP had a sensitivity of 70% (54 - 83%) equivalent to the overall sensitivity of sputum smear microscopy [49% (33 - 64%), p=0.064; Table 7]. LAMP sensitivity was similar in patients with CD4<200 cells/ml vs. CD4≥200 cells/ml [67% (82 - 96%) vs. 65% (41 - 83%); p=0.937, respectively]. In smear-negative patients, the sensitivity of LAMP was 44% (23 - 67%). The specificity of LAMP was 91% (82 - 96%) which was similar to smear microscopy [99% (92 - 99%); p=0.064]. The PPV of LAMP was similar to smear microscopy [81% (65 - 91%) vs. 95% (75 - 99%); p=0.207, respectively, Table 7], while the NPV was the same (85% vs. 78%; p=0.267) in LAMP and smear microscopy respectively.

Table 7. Performance outcomes of LAMP for the detection of MTB in persons infected with HIV, stratified by CD4 count. (Liquid culture positivity for *Mycobacterium tuberculosis* serves as a reference standard).

	HIV-infected patients **n=108**[*]		Patients infected with HIV with CD4 count < 200 cells/ml [+] **n=31**		Patients infected with HIV with CD4 count ≥ 200 cells/ml [β] **n=56**	
	sens. (95% CI)	**spec.** (95% CI)	**sens.** (95% CI)	**spec.** (95% CI)	**sens.** (95% CI)	**spec.** (95% CI)
Smear microscopy (%)	**49** (33-64) 18/37	**99** (92-99) 67/68	**44** (19-73) 4/9	**100** (85-100) 23/28	**41** (22-64) 7/17 (p=0.879)[+]	**97** (86-99) 35/36 (p=0.057)[+]
LAMP (%)	**70** (54-83) 26/37 (p=0.064)[ł]	**91** (82-96) 62/68 (p=0.064)[ł]	**67** (35-88) 6/9 (p=0.395)[ł]	**91** (72-97) 20/22 (p=0.417)[ł]	**65** (41-83) 11/17 (p=0.937)[+] (p=0.194)[ł]	**89** (75-96) 32/36 (p=0.842)[+] (p=.0.842)[ł]
LAMP performed in smear-negative individuals (%)	**44** (23-67) 7/16 (p=0.082)[≠]	**91** (82-96) 61/67 (p=0.979)[≠]	**50** (15-85) 2/4	**91** (72-97) 20/22 (p=0.999)[≠]	**40** (17-69) 4/10 (p=0.769)[+] (p=0.248)[≠]	**89** (74-95) 31/35 (p=0.816)[+] (p=0.816)[≠]
	PPV (95% CI)	**NPV** (95% CI)	**PPV** (95% CI)	**NPV** (95% CI)	**PPV** (95% CI)	**NPV** (95% CI)
Smear microscopy (%)	**95** (75-99) 18/19	**78** (68-85) 67/86	**100** (51-100) 4/4	**81** (63-92) 22/27	**88** (53-98) 7/8 (p=0.667)[+]	**78** (64-87) 35/45 (p=0.728)[+]
LAMP (%)	**81** (65-91) 26/32 (p=0.207)[ł]	**85** (76-91) 62/73 (p=0.267)[ł]	**75** (41-93) 6/8 (p=0.63)[ł]	**87** (68-94) 20/23 (p=0.63)[ł]	**73** (48-89) 11/15 (p=0.954)[+] (p=0.503)[ł]	**84** (70-93) 32/38 (p=0.799)[+] (p=0.478)[ł]

LAMP performed in smear-negative individuals (%)	**54** (29-77) 7/13 (p=0.082)$^{\neq}$	**87** (77-93) 61/70 (p=0.713)$^{\neq}$	**50** (15-85) 2/4	**91** (72-97) 20/22 (p=0.709)$^{\neq}$	**50** (22-78) 4/8 (p=0.999)$^{+}$ (p=0.311)$^{\neq}$	**84** (69-92) 31/37 (p=0.481)$^{+}$ (p=0.961)$^{\neq}$

Definition of abbreviations: CI; confidence interval, NPV; negative predictive value; PPV; positive predictive value.
***Excludes 21 HIV-positive patients with no CD4 count data.**
$^{+}$P-values marked with a $^{+}$ are for comparisons according to HIV-status.
† P-values marked with a † are for comparisons between LAMP and smear microscopy
‡ P-values marked with a ‡ are for comparisons between LAMP and LAMP performed in smear-negative patients

2.4.5. Culture time to positivity and LAMP performance

The graph, **Figure 12**, shows that as time to culture positivity increases, LAMP sensitivity decreases. LAMP-positivity had a significant shorter time to culture-positivity than LAMP-negative patients , where, the mean time to detection (IQR) for LAMP-positive patients was 16 days (4-50), while in LAMP-negative patients it was 40 days (6-50); $p<0.0001$.

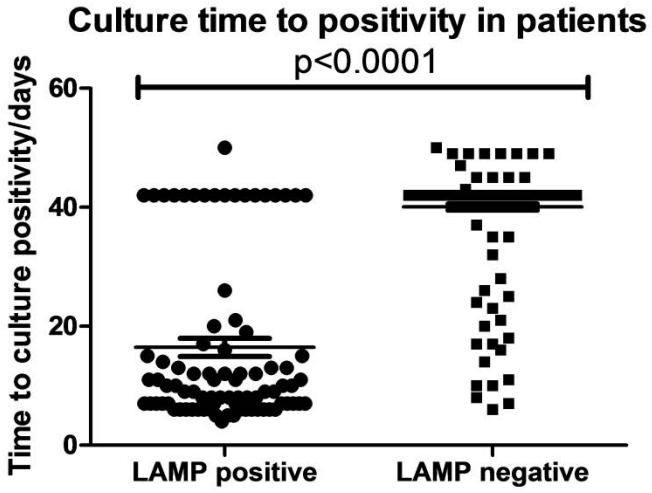


Figure 12: Graph showing culture time to positivity in patients who are LAMP-positive or -negative.

2.5. DISCUSSION

This study evaluated the LAMP diagnostic accuracy in a high prevalence setting. The key findings were that

i. The LAMP assay had modest sensitivity.

ii. LAMP performed modestly in HIV-infected individuals as compared to smear microscopy.

iii. LAMP has an incremental yield over smear microscopy.

With the existence of smear-negative TB and HIV co-infection in high HIV prevalent settings, sensitive NAATs such as LAMP can be used to diagnose TB suspects. In our study, the sensitivity in smear-negative cases was 48 % (32.4 - 65.7%; 14 of 29), which is similar with an evaluation study by Boehme *et al.* [65] of the LAMP prototype targeting the gyrB gene which had a sensitivity of 48.8% in smear-negative, culture- positive patients.

In a study by Pandey *et al.* [66], in Nepalese patients, their in-house LAMP assay had a sensitivity of 100% (96/96) while having a specificity of 94.2% (98/104). In our study, we observed different sensitivity having a sensitivity of 70%, but similar specificity of 91% to their findings. In India, [88] their LAMP assay showed a sensitivity of 44% and specificity of 94.4% which was way below our findings where we had sensitivity of 70%, however when they prolonged the incubation period to 90 minutes and 120 minutes, they experienced improved sensitivities (85.7% and 91.7%; respectively), and specificities of 93.9% and 90.9% respectively.

Notably, in a study by Nagdev *et al.* [89], in India, they showed a high sensitivity (88.23%) of their LAMP assay targeting the insertion sequence 6110 when diagnosing TBM using CSF which might otherwise show that LAMP performance in non sputum samples may perform better though further research is still needed to ascertain this. However, in these comparison studies [65, 66, 89, 90], the gold standard was culture positivity, similar to our reference standard in our study, whereas Nagdev *et al*. [89], had an additional reference that of clinical features such as sub-acute or chronic fever and signs of meningeal irritation for TBM positivity in addition to culture positivity. Amplicor (Roche Diagnostics, Basel Switzerland) which has a sensitivity of 92% in smear-positive, culture-positive specimens and 60% in smear-negative specimens shows considerable advantage over LAMP (77% and 48% respectively) but the only problem with this application is of infrastructure constraints, need for PCR space and the time period of at least 7 days taken until results are out especially in a high burden set up like where our research was conducted [91].

Also comparing LAMP with Xpert MTB/RIF and MTBDRplus, it has been observed that Xpert MTB/RIF has a greater sensitivity of 98.2% in smear-positive specimens, and 75.2% in smear-negative patients in a multi-country study by Boehme and colleagues [44], with 76% observed with the MTBDRplus assay amongst smear-positive, culture-positive patients in one study by Scott and colleagues [20]. Other studies have reported sensitivities of NAAT's between 69% to 85% in extra-pulmonary specimens [52]. However, the Xpert MTB/RIF system given its high operational costs and cold chain storage requirements for cartridges and Xpert buffers especially within high burdened resource limited set ups might face limited applicability while MTBDRplus and other associated NAAT's require separate steps

for sample (sputum) liquefaction, genomic DNA extraction, target amplification and amplicon detection, which eventually means that there is need for numerous steps, different work stations and increased risk of contamination with genomic bacterial DNA or amplicons generated [65]. The limitations for the comparison studies are that the test was not evaluated in smear-negative TB cases, which is an important group of patients while also repeated power supply and cold chain interruptions could have adversely impacted on the test's performance [65]. Important to note is the fact that the clinical follow-up of LAMP-positive, culture-negative patients was not done which could have allowed evaluation of the test's false-positive error rate [65]. The study by Pandey *et al*. [66] showed that the number of culture-positive and smear-negative samples was not large enough to strongly conclude superiority of the test to smear and comparable sensitivity to culture.

Inhibition might have been a reason for our study to have such modest sensitivity probably as a result of specimen morphological characteristics such as viscosity of sputum samples and whether the sample was bloody, which can impact on liquefaction and extraction protocols of the assay which we didn't assess which might affect the performance of the test.

The general observation of low specificity (Table 6 and Table 7), might probably be as a result of general cross contamination caused by lack of proper cleaning, and disposition of contaminated reagents and or surfaces which has to be strictly adhered to in order to get rid of amplicons. Also technical issues like defective heating tubes and absorbent tubes can ultimately influence such problems of low specificity.

This particular study had a limited sample size as only the Cape Town site specific data was used for analysis, while also sampling bias could have ensured as a result of the difficulty in comparison between subgroups.

However, some of the limitations of the LAMP test are that it has poor sensitivity with paucibacillary specimens such as smear-negative and extra pulmonary samples. This is complicated by the mycobacterial cell wall physiology which makes it difficult to extract DNA from cells while the assay also has the disadvantage of being unable to detect drug resistance.

Overall, use of the LAMP test is feasible as a NAAT that allows direct identification of *M. tuberculosis*. Due to its ease of use in developing countries, coupled with its

high specificity and modest sensitivity, it can lead to the identification of *M. tuberculosis* in smear-positive individuals, and also confirmation of diagnosis in suspected smear- negative TB cases.

Conclusion

LAMP modestly out performed smear microscopy and can rapidly increase the case detection of TB in developing countries. The LAMP assay appears to be a promising rule-in test, with a specificity of 91%, when using sputum from patients with suspected TB. However, although the sensitivity (70%) is higher than that of smear microscopy, the LAMP assay in this study did not have incremental value over smear (difference between the sensitivities was not statistically significant). It should be emphasized that the test isn't ready for use yet given the high false positive error rate (specificity < 95%), while these are preliminary data, further development maybe required on the stability and sensitivity of LAMP for its application in clinical diagnosis of TB.

3. CHAPTER 3

THE DIAGNOSTIC ACCURACY OF XPERT MTB/RIF PERFORMED ON NON-SPUTUM SPECIMENS FROM PATIENTS SUSPECTED OF TB.

3.1. Xpert MTB/RIF performance using bronchoalveolar lavage fluid (BALF) in diagnosing smear-negative and or sputum scarce TB.

ABSTRACT

Background

Current tools for TB diagnosis pose several challenges such as suboptimal accuracy, slow test turn-around time especially at point-of-care, and lack of simplicity, and ease of operation. Smear-negative and sputum-scarce TB coupled with HIV co-infection further compromises the accuracy of existing tools. This study evaluated the diagnostic accuracy of Xpert MTB/RIF on broncho-alveolar lavage fluid (BALF) collected from South African patients.

Methods

152 patients who were sputum scarce or smear-negative were recruited into the study. 1 ml of the BALF aliquot or a re-suspended pellet from 10 ml of the broncho-alveolar lavage fluid obtained from each patient was used for Xpert testing. Liquid culture was performed on BALF collected at the same time as that used for Xpert testing, where liquid culture served as a reference standard for definite TB. We evaluated accuracy according to HIV and smear microscopy status, when these data were available. Also, the relationship between Xpert performance and culture time to positivity (TTP) was compared.

Results

27 of 152 patients were culture-confirmed for *M. tuberculosis*. Of the definite TB patients, Xpert significantly outperformed smear microscopy (93%; 95% CI 77 - 98% versus 58%; 95% CI 39 - 74%; **p<0.004**, respectively). Centrifugation of BALF

though not significant, slightly increased Xpert sensitivity to 95% (75 - 99%). The time to culture-positivity (TTP) was significantly influenced by the HIV-status of patients with mean TTP values (23.58 vs. 21.48 days; p=0.02), while there was no correlation between Xpert MTB/RIF generated cycle threshold values (C_T) and TTP, p=0.09.

Conclusion

Xpert MTB/RIF significantly outperformed smear microscopy on BALF and has excellent performance in diagnosing sputum-scarce or smear-negative HIV-coinfected TB cases.

Research Outputs:

Grant Theron, Jonny Peter, Richard Meldau, Hoosain Khalfey, Phindile Gina, ***Brian Matinyenya****, Laura Lenders, Gregory Calligaro, Brian Allwood, Gregory Symons, Ureshnie Govender, Mashiko Setshedi & Keertan Dheda (2013). Accuracy and impact of Xpert MTB/RIF for the diagnosis of smear-negative or sputum-scarce tuberculosis using bronchoalveolar lavage fluid.* Thorax, 2013. **68** (11): p. 1043-51.

Grant Theron, Jonny Peter, Greg Calligaro, Richard Meldau, Colleen Hanrahan, Hoosain Khalfey, ***Brian Matinyenya****, Tapuwa Muchinga, Liezel Smith, Shaheen Pandie, Laura Lenders, Bongani M. Mayosi, Vinod Patel & Keertan Dheda (2014). Determinats of PCR performance (Xpert MTB/RIF), includng bacterial load and inhibition, for TB diagnosis using specimens from different body compartments. Sci Rep, 2014.* ***4****: p. 5658.*

3.1.1. INTRODUCTION

The current methods of diagnosing TB such as smear microscopy and or cultures either have poor sensitivity or are time consuming, which has adverse effects on patient treatment outcomes. However, obtaining a high quality biological specimen for testing is paramount to reducing the diagnostic delay [57].

Sputum scarce TB, smear-negative TB and the difficulty associated with diagnosing extrapulmonary tuberculosis (EPTB), all mean that other biological fluids other than sputum should be evaluated in determining the diagnostic accuracy of NAATs especially in TB endemic areas.

Smear-negative TB disease increases morbidity and mortality and in cases of HIV co-infection, smear microscopy becomes problematic as it misses a considerable number of cases. For example, Behr *et al*, found that approximately 17% of TB transmission is as a result of smear-negative TB cases [92]. Also important to note is the fact that acid-fast bacilli smear microscopy for detecting TB is limited by the need for 5000-10000 bacilli per millilitre [93]. In a study in Vietnam by Nhu *et al* [94], smear microscopy performed in patients with suspected TB-meningitis on CSF had a sensitivity of 79% (72 - 84%; 143/182). This necessitates the need for improved point of care diagnosis, which is accurate and rapid.

Bronchoscopy with BALF or bronchial washings is often performed in patients who are smear- negative and or sputum scarce as this group of patients represents up to a third of those with pulmonary TB [57]. Xpert MTB/RIF has an improved diagnostic accuracy over smear microscopy [44], while it still has a detection deficit in smear-negative and HIV-infected patients [95].

There are limited published studies which evaluated Xpert MTB/RIF assay's performance on BALF [54, 96]. We prospectively evaluated the accuracy of Xpert MTB/RIF performed on BALF obtained from smear-negative or sputum scarce patients suspected of having TB recruited from a hospital in Cape Town, South Africa.

3.1.2. HYPOTHESIS, AIMS AND OBJECTIVES

Hypothesis

The Xpert MTB/RIF is an accurate point of care TB diagnostic tool using BALF as a biological sample which will improve diagnosis in smear-negative and or sputum scarce TB patients.

Aims

To determine the performance outcomes of Xpert MTB/RIF on BALF from TB suspects at a hospital in Cape Town, South Africa vs. smear microscopy and liquid culture techniques for the diagnosis of pulmonary TB.

Objective

- To determine the diagnostic accuracy of Xpert MTB/RIF in smear-negative and -positive patients.
- To determine Xpert MTB/RIF performance in HIV-infected and -uninfected patients.

3.1.3. MATERIALS AND METHODS

3.1.3.1. Study design

This study was prospectively carried out to determine the performance of Xpert MTB/RIF in patients with symptoms of pulmonary TB who were referred to the respiratory clinic at Groote Schuur hospital in Cape Town, South Africa. Initially the project included the collection of BALF which was frozen at -20°C for later testing, which was designated phase 1 and was for research purposes only. Treatment was guided by smear microscopy and culture results in phase 1. Phase 2 included collection of this BALF sample which was used immediately for Xpert MTB/RIF testing and in this phase used for guiding treatment in patients.

3.1.3.2. Study Flow

Detailed patient and laboratory specific information was recorded and patients underwent HIV testing upon counselling. An aliquot of BALF was either frozen at -20°C for later batched testing in phase one, or used immediately for MTB/RIF testing in phase two. Patients were classified as definite TB if they had at least one specimen culture- positive for TB or upon histological investigation of the transbronchial biopsy were reported to contain caseating or necrotising granulomatous inflammation consistent with TB [57], while those patients with BALF which was culture-negative for TB and the absence of histological evidence of active TB disease were classified as probable TB ,if initiated on anti-TB treatment. Empirical treatment of a patient was initiated by the attending physician in the absence of a positive test result.

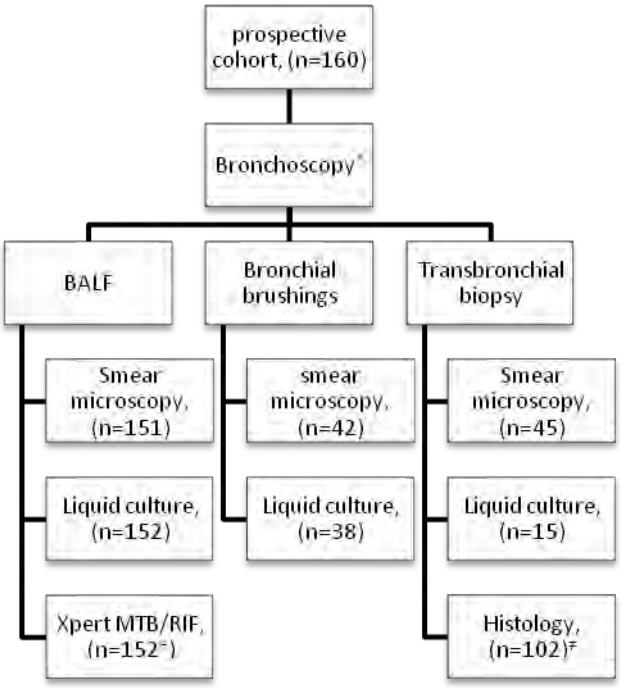


Figure 13: Study flow diagram showing the patients included in the analysis and test results.

Definition of abbreviations. BALF, bronchoalveolar lavage fluid; PBS, phosphate buffer saline.

*All patients provided BALF. Bronchial brushings and biopsies were provided at the discretion of the attending clinician to the reference laboratory. Smear and culture were performed on these latter two specimens when possible by the reference laboratory.

+MTB/RIF was performed on fresh BALF from 74 patients. Fluid thawed from a frozen specimen was used for MTB/RIF for 78 patients recruited prior to the WHO endorsement of Xpert MTB/RIF.

#Histology was considered compatible with active TB if necrotising or caseating granulomatous inflammation were observed by the reference laboratory. Eleven individuals had record of histology information missing

3.1.3.3. Sample size

In total 160 patients were enrolled with suspicion of TB. Each patient provided BALF for analysis.

3.1.3.4. Inclusion criteria

Patients above 18 years of age were recruited. Patients should have had at least one symptom of TB, a chest radiograph with infiltrates compatible with active TB and or at least two consecutive sputum smear microscopy results negative for AFB(within two weeks of enrolment) and or were unable to self-expectorate sputum.

3.1.3.5. Subject Recruitment

Patients who had symptoms consistent with PTB presenting to the hospital were asked to participate. Patients were told that participation was voluntary and that they had the opportunity to ask questions individually. A consent form was signed by all participating patients. Eligible patients who signed the informed consent form had their medical history taken, and underwent a clinical examination, HIV testing and bronchoscopy after referral. . Samples were collected before the patient started TB treatment. All results were recorded in the Case Report Form (CRF), which was entered into an electronic database. Study participants with incomplete CRF's were withdrawn from the study and new study subjects enrolled instead. Statistical analysis was done using a statistical software; OpenEpi (Open Source Epidemiologic Statistics for Public Health, Version 2.3. www.OpenEpi.com) and Graphpad Prism (version 5).

3.1.3.6. Laboratory Based Methods

Bronchoscopy was carried out by trained pulmonologists. BALF collected was split and one aliquot sent immediately to Groote Schuur Hospital for NaLC-NaOH decontamination, and the second aliquot was used for MTB/RIF testing. The decontaminated specimen was examined twice by concentrated fluorescence smear microscopy and liquid culture for MTB using BACTEC MGIT 960 System (BD Diagnostics, USA) [97]. Culture-positive isolates underwent routine phenotypic drug susceptibility testing for rifampicin and isoniazid using the MGIT 960 SIRE kit. Upon request, concentrated fluorescence smear microscopy and liquid culture were also performed on bronchial washings and transbronchial biopsy specimens (where transbronchial biopsies underwent histological examination if available) [57]. The assay was performed on 1 ml of uncentrifuged BALF when available. The supernatant

was decanted, and the pellet re-suspended in 1 ml of sterile phosphate- buffered saline (PBS) and Xpert MTB/RIF procedure was then followed as described [47, 98], with the laboratory technician performing the procedure blinded to patient information and test results. Phase two Xpert MTB/RIF results were reported immediately to clinical staff.

3.1.4. RESULTS

3.1.4.1. Study population and TB Diagnoses

160 patients with suspected pulmonary TB who were referred for bronchoscopy consented to participate in the study and were recruited with eight patients being excluded from the analysis (Figure 13). Of the 152 included patients, 110 (72%) were sputum-scarce and 44 (29%) were HIV- infected (Table 8). 27 of the 152 patients (18%) were BALF culture-positive for *M. tuberculosis*. When a bronchial brushing or transbronchial biopsy was cultured, 9 out of 38 patients (24%) and 4 out of 15 (27%) of patients were positive respectively. However, all culture-positive bronchial brushings and or transbronchial biopsies had culture-positive BALF. Transbronchial biopsies histological examination revealed active TB compatibility in 7 of 102 cases (7%) that were examined, with one patient having culture-negative BALF.

Table 8. Demographic and clinical characteristics

Demographic/Clinical characteristic	Cohort (n=152)	BALF culture-positive (n=27)	BALF culture-negative (n=125)	p value
Median age in yrs	46.1	41.4	45.9	0.418
Male (%)	82 (54)	10 (37)	72 (57)	0.621
Female (%)	70 (46)	17 (63)	53 (42)	0.057
Sputum - scarce (%)	110 (72)	19 (70)	91 (73)	0.788
HIV infected*	44 (29)	8 (30)	36 (29)	0.918
Median CD4 count (cells/µl) (IQR) if HIV infected ł	165 (67-406)	151 (58-665)	179 (67-406)	0.698
Previous TB treatment≠ (%)	50 (33)	5 (19)	45 (36)	0.078
Smoker (past or current) α (%)	41 (27)	5 (19)	36 (29)	0.287

***Twenty-four patients were of unknown HIV status (test refused or data missing).**

łTen patients with HIV infection were missing CD4 count data.
≠six patients were missing previous TB data.
αTwelve patients were missing smoking data.
BALF, bronchoalveolar lavage fluid; TB, tuberculosis
(P- value comparisons are between BALF culture-positive vs. BALF culture-negative patients)

3.1.4.2 **Diagnostic accuracy of Xpert MTB/RIF performed on BALF**

Of the 27 definite TB (culture-positive) patients in our cohort, 25 had Xpert-positive BALF. Xpert had a sensitivity of 93% (25/27; 77 - 98%) which was significantly higher than smear microscopy performed on the same BALF, 58% (15/26; 39- 74.5%; p<0.004; Table 9). 5 of the 127 non-TB cases (culture-negative) had Xpert-positive BALF having a specificity of 96% (120/125, 91- 98%) similar to smear microscopy which had, 99.2% (124/125; 96 - 99%; p=0.121). The positive predictive value (PPV) of Xpert was 83% (64 - 93%; 25/30) which was similar to that of smear microscopy, 94% (72 - 99%; 15/16; p=0.370; Table 9), while their (Xpert MTB/RIF and smear microscopy) negative predictive values (NPV) were not similar [98% (94 - 100%; p=0.018) vs. 92% (86 - 95%), respectively, Table 9].

3.1.4.2. Impact of BALF processing on Xpert MTB/RIF performance

In this cohort, 98 patients had BALF available for centrifugation, where 19 patients were culture-positive for TB. We found that centrifugation resulted in a slight increase in Xpert MTB/RIF sensitivity, 95% (75 - 99%). Also centrifugation of BALF increased Xpert MTB/RIF sensitivity in HIV-infected patients having sensitivity of 100% (3/3; 44% - 100%), whereas for uncentrifuged BALF the sensitivity was 75% (6/8; 41% - 93%). However, it should be noted that this increase in sensitivity was not significant (i.e. 100% vs. 75%; p=0.509).

3.1.4.3. Accuracy of Xpert MTB/RIF in HIV co-infected patients

Xpert had a significant increase in sensitivity of 100% (12/12, 76 - 100%, p=0.018) in HIV-uninfected individuals vs. smear microscopy, 58% (7/12; 32 - 81%), whereas in HIV-infected patients, the assay had modest sensitivity of 75% (6/8; 41 - 93%; Table 9). This drop in sensitivity was not significant in Xpert MTB/RIF performance (100%

vs. 75%; p=0.147) when performed in these particular cluster of patients (HIV-infected and uninfected patients).

3.1.4.4. Xpert MTB/RIF performance in smear-negative cases

It should be noted that Xpert MTB/RIF had a sensitivity of 82% (52 - 95%), while it also retained a higher specificity of 97%. In HIV-uninfected patients who were smear-negative for TB, the assay had 100% sensitivity (57-100%), and sensitivity dropped to 33% (6 -79%; 1/3; p=0.11, Table 9) in HIV-infected patients.

Table 9. Accuracy of Xpert MTB/RIF performed on BALF and stratified by HIV status of patients. (Liquid culture positivity for *M tuberculosis* serves a reference standard).

	All patients (n=152)		**HIV-uninfected (n=84)**		**HIV-infected (n=44)**	
	sens. (95% CI) n/N	**spec.** (95% CI) n/N	**sens.** (95% CI) n/N	**spec.** (95% CI) n/N	**sens.** (95% CI) n/N	**spec.** (95% CI) n/N
Conc. smear microscopy (%)	**58** (39-75) 15/26	**99** (96-100) 124/125	**58** (32-81) 7/12	**100** (95-100) 72/72	**57** (25-84) 4/7 (p=0.960)$^{+}$	**97** (86-100) 35/36 (p=0.333)$^{+}$
MTB/RIF performed on uncentrifuged BALF (%)	**93** (77-98) 25/27 **(p=0.004)$^{\text{I}}$**	**96** (91-98) 120/125 (p=0.121)$^{\text{I}}$	**100** (76-100) 12/12 **(p=0.018)$^{\text{I}}$**	**96** (86-99) 69/72 (p=0.122)$^{\text{I}}$	**75** (41-93) 6/8 (p=0.147)$^{+}$ (p=0.527)$^{\text{I}}$	**94** (82-99) 34/36 (p=0.747)$^{+}$ (p=0.62)$^{\text{I}}$
MTB/RIF performed on uncentrifuged BALF in smear- negative patients (%)	**82** (52-95) 9/11 (p=0.391)$^{\neq}$	**97** (92-99) 120/124 (p=0.759)$^{\neq}$	**100** (57-100) 5/5	**96** (89-99) 69/72 (p=0.998)$^{\neq}$	**33** (6-79) 1/3 (P=0.107)$^{+}$ (p=0.303)$^{\neq}$	**97** (86-100) 34/35 (p=0.803)$^{+}$ (p=0.636)$^{\neq}$
MTB/RIF performed on a re-suspended pellet (centrifuged BALF) (%)	**95** (75-99) 18/19 (p=0.824)$^{\beta}$	**99** (93-100) 71/72 (p=0.349)$^{\beta}$	**100** (76-100) 12/12	**95** (84-99) 40/42 (p=0.871)$^{\beta}$	**100** (44-100) (3/3) (p=0.509)$^{\beta}$	**86** (65-95) 18/21 (p=2402)$^{\alpha}$ (p=0.313)$^{\beta}$
	PPV+ (95% CI) n/N	**NPV+** (95% CI) n/N	**PPV+** (95% CI) n/N	**NPV+** (95% CI) n/N	**PPV+** (95% CI) n/N	**NPV+** (95% CI) n/N
Conc. smear microscopy (%)	**94** (72-99) 15/16	**92** (86-95) 124/135	**100** (65-100) 7/7	**94** (86-97) 72/77	**80** (38-96) 4/5	**92** (79-97) 35/38

MTB/RIF performed on uncentrifuged BALF (%)	**83** (64-93) 25/30 (p=0.370)[ł]	**98** (94-100) 120/122 **(p=0.018)**[ł]	**80** (55-93) 12/15 (p=0.296)[ł]	**100** (95-100) 69/69 **(p=0.038)**[ł]	**75** (41-93) 6/8 (p=0.794)[+] (p=0.881)[ł]	**94** (82-99) 34/36 (p=0.115)[+] (p=0.722)[ł]
MTB/RIF performed on uncentrifuged BALF in smear- negative patients (%)	**69** (42-87) 9/13 (p=0.333)[≠]	**98** (94-100) 120/122	**63** (31-86) 5/8 (p=0.415)[≠]	**100** (95-100) 69/69	**50** (10-91) 1/2 (p=0.800)[+] (p=0.600)[≠]	**94** (82-99) 34/36 (p=0.115)[+]
MTB/RIF performed on a re-suspended pellet (%)	**95** (75-99) 18/19 (p=0.279)[β]	**99** (93-99) 71/72 (p=0.936)[β]	**86** (60-96) 12/14 (p=0.721)[β]	**100** (91-100) 40/40	**50** (19-81) 3/6 (p=0.145)[+] (p=0.406)[β]	**100** (69-96) 18/18 (p=0.440)[β]

+Based on TB prevalences of 18%, 14% and 18% overall and in patient groups with HIV infection and without infection. BALF, bronchoalveolar lavage fluid; NPV, negative predictive value; PPV, positive predictive value; TB, tuberculosis.
[+]P-values marked with a[+] are for comparisons according to HIV-status.
[ł]P-values marked with a[ł] are for comparisons between Xpert MTB/RIF and smear microscopy.
[≠]P-values marked with a[≠] are for comparisons between Xpert MTB/RIF and Xpert performed in smear-negative patients.
[β]P-values marked with a[β] are for comparisons between Xpert MTB/RIF centrifuged and Xpert MTB/RIF uncentrifuged.

3.1.4.5. Diagnostic accuracy of Xpert MTB/RIF performed on uncentrifuged BALF stratified by the CD4 counts of patients

In this cohort, of the eight definite TB cases (culture-positive), six had Xpert-positive BALF. Xpert MTB/RIF test had a sensitivity of 75% (41 - 93%; 6/8), which was similar to smear microscopy performed on the same BALF, 57% (25- 84%; p=0.527), table 3.3. Two of the thirty-six non TB cases had Xpert -positive BALF resulting in specificity of 94% (82 - 99%); which was similar to smear microscopy [97%; (86 - 100%; p= 0.62), Table 10]. The PPV and NPV of Xpert were 75% (41 - 93%) and 94% (82 - 94%), respectively.

3.1.4.6. Effect of BALF centrifugation on Xpert MTB/RIF performance in HIV-infected patients stratified by CD4 counts

Centrifugation of the BALF resulted in Xpert having a sensitivity of 100% (68-100%) in patients with CD4 counts less than 200 cells/ml, compared to 73% (43-90%; p=0.170, Table 10) in patients with CD4 counts greater than or equal to 200 cells/ml.

Table 10. Performance outcomes of Xpert MTB/RIF on BALF for the detection of MTB in persons infected with HIV, stratified by CD4 count. (Liquid culture positivity for *Mycobacterium tuberculosis* serves as a reference standard).

	HIV-infected patients n=44		**Patients infected with HIV with CD4 count < 200 cells/ml n=18**		**Patients infected with HIV with CD4 count ≥ 200 cells/ml n=16**	
	sens. (95% CI)	**spec.** (95% CI)	**sens.** (95% CI)	**spec.** (95% CI)	**sens.** (95% CI)	**spec.** (95% CI)
Smear microscopy (%)	**57** (25-84) 4/7	**97** (86-100) 35/36	**67** (21-94) 2/3	**93** (70-99) 14/15	**-**	**100** (51-100) 4/4 (p=0.429)$^{+}$
Xpert MTB/RIF performed on uncentrifuged BALF (%)	**75** (41-93) 6/8 (p=0.527)^{ł}	**94** 82-99 34/36 (p=0.620)^{ł}	**100** (44-100) 3/3 (p=0.500)^{ł}	**93** (70-99) 14/15 (p=0.998)^{ł}	**50** (10-91) 1/2 (p=0.400)$^{+}$	**100** (99-100) 14/14 (p=0.517)$^{+}$
MTB/RIF performed on uncentrifuged BALF in smear negative patients (%)	**33** (6-79) 1/3 (p=0.303)$^{\neq}$	**97** (86-100) 34/35 (p=0.636)$^{\neq}$	**100** (21-100) 1/1	**100** (79-100) 14/14 (p=0.998)$^{\neq}$	**-**	**100** (79-100) 14/14
MTB/RIF performed on a re-suspended pellet (centrifuged BALF) (%)	**100** (44-100) 3/3 (p=0.509)$^{\beta}$	**86** (65-95) 18/21 (p=0.313)$^{\beta}$	**100** (34-100) 2/2	**100** (68-100) 8/8 (p=0.652)$^{\beta}$	**-**	**73** (43-90) 8/11 (p=0.170)$^{+}$ (p=0.170)$^{\beta}$
	PPV (95% CI)	**NPV** (95% CI)	**PPV** (95% CI)	**NPV** (95% CI)	**PPV** (95% CI)	**NPV** (95% CI)

Smear microscopy (%)	**80** (38-96) 4/5	**92** (79-97) 35/38	**67** (21-94) 2/3	**93** (70-99) 14/15	**-**	**80** (38-96) 4/5 (p=0.500)$^{+}$
Xpert MTB/RIF performed on uncentrifuged BALF (%)	**75** (41-93) 6/8 (p=0.881)^{l}	**94** (82-99) 34/36 (p=0.722)^{l}	**75** (30-95) 3/4 (p=0.857)^{l}	**100** (79-100) 14/14 (p=0.998)^{l}	**100** (21-100) 1/1	**93** (70-99) 14/15 (p=0.998)$^{+}$ (p=0.500)^{l}
MTB/RIF performed on uncentrifuged BALF in smear negative patients (%)	50 (10-91) 1/2 (p=0.600)$^{\neq}$	94 (82-99) 34/36 (p=0.998)$^{\neq}$	100 (21-100) 1/1 (p=0.800)$^{\neq}$	100 (21-100) 14/14	**-**	93 (70-99) 14/15 (p=0.998)$^{+}$ (p=0.998)$^{\neq}$
MTB/RIF performed on a re-suspended pellet (centrifuged BALF) (%)	**50** (19-81) 3/6 (p=0.406)$^{\beta}$	**100** (82-100) 18/18 (p=0.440)$^{\beta}$	**100** (34-100) 2/2 (p=0.667)$^{\beta}$	**100** (68-100) 8/8 (p=0.998)$^{\beta}$	**-**	**100** (68-100) 8/8 (p=0.652)$^{\beta}$

+Based on TB prevalences of 18%, 14% and 18% overall and in patient groups with HIV infection and without infection. BALF, bronchoalveolar lavage fluid; CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value; TB, tuberculosis.

$^{+}$P-values marked with a^{+} are for comparisons according to HIV-status.

^{l}P-values marked with a^{l} are for comparisons between Xpert MTB/RIF and smear microscopy.

$^{\neq}$P-values marked with a$^{\neq}$ are for comparisons between Xpert MTB/RIF and Xpert performed in smear-negative patients.

$^{\beta}$P-values marked with a$^{\beta}$ are for comparisons between Xpert MTB/RIF centrifuged and Xpert MTB/RIF uncentrifuged.

3.1.4.7. Markers of bacterial load (TTP's),HIV status and Xpert MTB/RIF performance

Xpert MTB/RIF-positivity and -negativity was similar to liquid culture time to positivity. The mean time to detection (IQR) for Xpert MTB/RIF-positivity was 20 days (0-49), whereas for Xpert MTB/RIF-negativity was [17 days (0-36); p=0.8997,

Figure 14]. HIV-positivity of patients significantly influenced liquid culture time to positivity; with HIV-positivity having mean time to detection (IQR) [23 days (0-42)]; while HIV-negativity was [21 days (0-136); p=0.023, Figure 15].

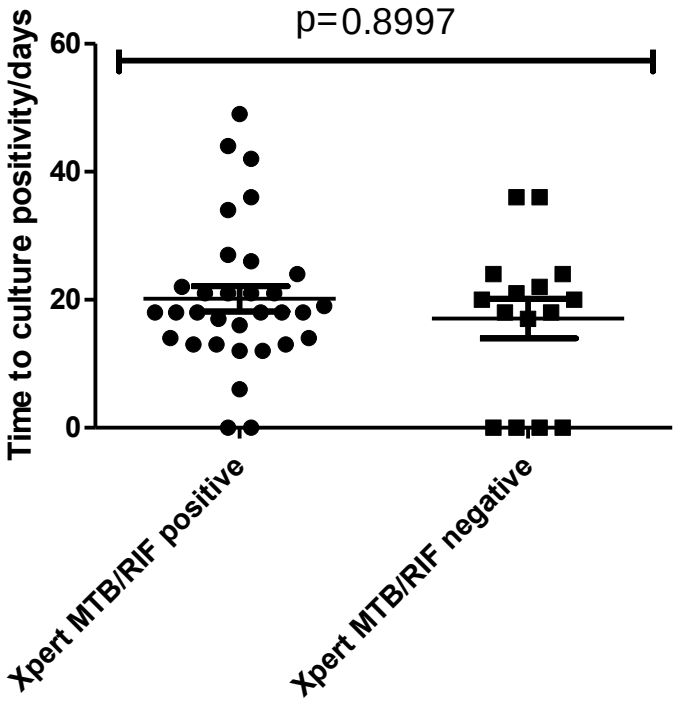


Figure 14: Comparison of time to culture positivity in Xpert MTB/RIF-positive and -negative patients.

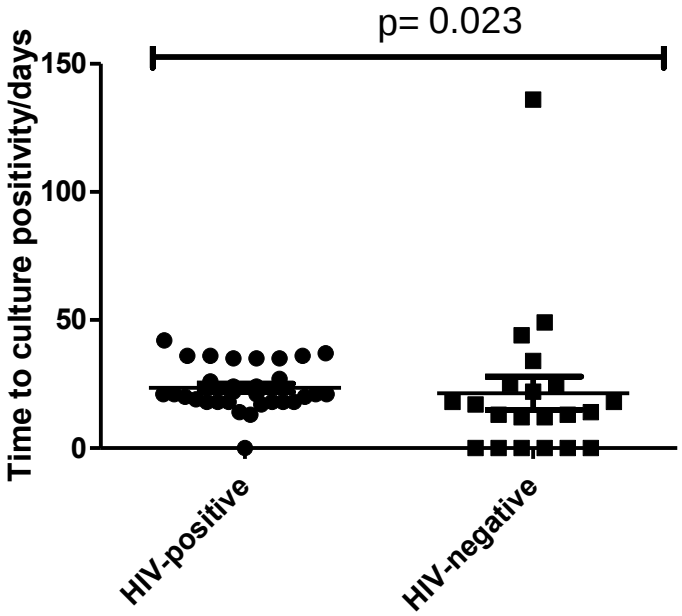


Figure 15: Comparison of time to culture positivity in HIV-infected and -uninfected patients.

3.1.4.8. Relationship of Xpert MTB/RIF-generated C_T (cycle threshold) values with bacterial load

There was no correlation between Xpert MTB/RIF-generated C_T values and TTP; (r^2=0.654; p=0.097; Figure 16).

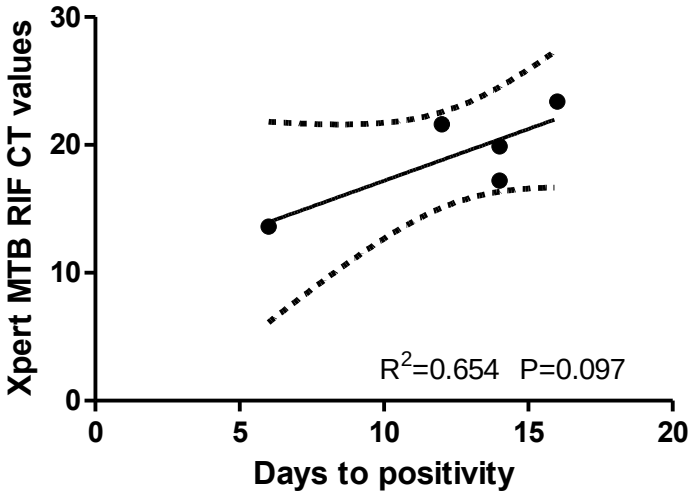


Figure 16: Correlation of BALF Xpert MTB/RIF cycle threshold values and BACTEC MGIT 960 time to culture positivity.

3.1.5. DISCUSSION

Studies showing the accuracy of Xpert on BALF are limited, and the impact of factors such as biological specimen processing, and HIV co-infection is unknown. The key findings of this study were that Xpert MTB/RIF significantly outperformed smear microscopy using BALF, while BALF centrifugation did not improve the diagnostic accuracy of Xpert MTB/RIF.

Information regarding Xpert MTB/RIF assay performance on non-sputum samples is emerging, though not extensively and sufficiently studied in high HIV-prevalent settings [99]. It has been noted that studies examining Xpert MTB/RIF performance in BALF [49, 96, 100-102] have been conducted; however, due to limited numbers of specimens, and the decreased number of TB cases, such information is limited.

In our study with a patient cohort of 152 patients, Xpert MTB/RIF sensitivity was 94%, having similar sensitivities to other respiratory specimens, such as in a study by Teo *et al*. [103], Xpert MTB/RIF sensitivity was found to be 90% (80- 96%). In another study in France [49], the test exhibited a sensitivity of 100% in smear-positive specimens irrespective of the site where the clinical specimen was obtained. In a similar study, Miller *et al*. [100] showed that the test had 100% sensitivity in smear-positive, culture-positive pulmonary specimens. Similarly, in a study in Turkey, Zeka *et al*. [104], showed that Xpert MTB/RIF test with smear-and culture-positive pulmonary specimens was 100% with a specificity of 98.3. Thus it can be concluded herein that our study findings are in accordance with prior published data signifying Xpert MTB/RIF performance in diagnosing sputum scarce or smear-negative TB patients.

The predominantly high sensitivities earlier mentioned, are markedly exceeding sensitivities reported for commercially available NAAT's. A study by Davis *et al*. [96], in Uganda, reported a sensitivity of 39% (28- 51%) and a specificity of 95% exhibited by the MTD test when mycobacterial culture was used as a reference standard. In a study in France, by Simonnet *et al. [101]*, they reported sensitivity of 100% in respiratory specimens and a specificity of 100% using the EMEQ (easyMAG/easyQ, bioMérieux, France) assay. Chou *et al*. [102], in Taiwan, showed that the Gen-Probe AMTD test had a sensitivity and specificity of 80% and 97.5% respectively in smear-negative pulmonary TB suspects.

In our study we found that five patients were Xpert MTB/RIF-positive but having culture-negative results, while four were smear-negative and one smear-positive in this group of Xpert MTB/RIF-positive patients. The markedly increased sensitivity, 93% of Xpert MTB/RIF on BALF in our study, was similar to a study on the performance of Xpert MTB/RIF system on sputum samples which Xpert MTB/RIF has been optimized for in definite TB patients having 90% sensitivity [44].

In one study it was shown that centrifugation of urine improved the diagnostic yield when Xpert MTB/RIF test was used in HIV-infected, sputum scarce patients with CD4 counts less than or equal to 200 cells/ml [36]. However, the same was not found to be consistent in our study when BALF was used, as we did not find a significant improvement in sensitivity when a similar volume of BALF was used.

We have also shown that Xpert MTB/RIF test had reduced or rather somewhat modest sensitivity of 75% in HIV-infected individuals. This might be attributable to the findings in one study that HIV infection is associated with a decreased likelihood of a positive Xpert MTB/RIF result in pulmonary specimens [99]. This is also in accordance with our study findings where Xpert MTB/RIF test results were not influenced by the bacterial burden of the specimen probably due to the decreased bacillary load in the lungs of HIV co-infected patients with PTB due to the lower frequency of cavitation in these patients [99].

Some of the limitations of this study are that we did not look at the use of induced sputum on the performance of Xpert which might influence the need for not using the assay in sputum scarce TB patients since the procedure (bronchoscopy) is expensive and demands expertise which might not be necessarily available in high burdened resource limited settings. Our data might be mostly relevant to low burden resource-advantaged settings experiencing an influx of migrants coming from high burdened set ups as such countries due to the availability of resources can afford bronchoscopy procedures. Another drawback to our study might be that the volume of BALF used might have been insufficient especially after parts of the BALF would have been sent for routine diagnostic testing. The number of patients involved in the study might have been too small to successfully arrive at a valid conclusion giving rise to sampling bias.

Conclusion

Xpert MTB/RIF system on BALF has excellent performance in diagnosing sputum scarce, and or smear-negative HIV co-infected TB cases in high HIV prevalent settings.

3.2. DIAGNOSTIC ACCURACY OF XPERT MTB/RIF USING TRACHEAL ASPIRATES IN DIAGNOSING PULMONARY TB.

ABSTRACT

Background

The Xpert MTB/RIF accuracy data using tracheal aspirates in suspected TB patients who are mechanically ventilated in the intensive care unit (ICU) is limited.

Methods

120 South African ICU patients suspected of TB were mechanically ventilated. Approximately 3 ml to 15 ml of tracheal aspirate secretions was collected, where 1 ml of the tracheal aspirate was aliquoted for Xpert testing. Liquid culture was performed on tracheal aspirates collected at the same time as that used for Xpert testing, where liquid culture served as a reference standard for definite TB. We evaluated diagnostic accuracy according to HIV and smear microscopy status, when these data were available. Also, the relationship between Xpert performance and TTP was compared.

Results

Of the 120 patients, only 71 had data that could be analysable. 11 of 71 patients had culture confirmed TB. For these culture confirmed TB cases, Xpert had a sensitivity of 91% (62-98%), whereas smear microscopy had a sensitivity of 55% (28-79%). The difference in sensitivity was not significant (91% vs. 55%, p=0.08) though the sample size was limited. There was no correlation between Xpert MTB/RIF generated C_T values and liquid culture TTP; p=0.08.

Conclusion

Xpert MTB/RIF outcompeted smear microscopy and is a potentially useful tool for the diagnosis of TB as a rule-out test in HIV-uninfected and or –infected patients.

Research outputs:

Grant Theron, Jonny Peter, Greg Calligaro, Richard Meldau, Colleen Hanrahan, Hoosain Khalfey, ***Brian Matinyenya****, Tapuwa Muchinga, Liezel Smith, Shaheen Pandie, Laura Lenders, Bongani M. Mayosi, Vinod Patel & Keertan Dheda (2014). Determinats of PCR performance (Xpert MTB/RIF), including bacterial load and inhibition, for TB diagnosis using specimens from different body compartments. Sci Rep, 2014.* ***4****: p. 5658.*

Gregory L Calligaro, Grant Theron, Hoosain Khalfey, Jonathan Peter, Richard Meldau, ***Brian Matinyenya****, Malika Davids, Liezel Smith, Anil Pooran, Maia Lesosky, Aliasgar Esmail, Malcolm G Miller, Jenna Piercy, Lancelot Michell, Rodney Dawson, Richard I Raine, Ivan Joubert, Keertan Dheda (2015). Burden of tuberculosis in intensive care units in Cape Town, South Africa, and assessment of the accuracy and effect on patient outcomes of the Xpert MTB/RIF test on tracheal aspirate samples for diagnosis of pulmonary tuberculosis: a prospective burden of disease study with a nested randomised controlled trial. Lancet Respir Med, 2015. 3(8): p. 621-30.*

3.2.1. INTRODUCTION

The incidence of PTB in the intensive care units (ICU) is poorly studied and the utility of Xpert MTB/RIF using tracheal aspirates hasn't been documented. Tools for the diagnosis of active TB disease include clinical suspicion, response to treatment, chest radiographs, staining for acid-fast bacilli (AFB), culture for mycobacteria and nucleic acid amplification assays [93]. The most common testing mode of TB in resource poor countries which are highly TB burdened is sputum smear microscopy, which has amongst its disadvantages having an ability to detect approximately only 50% of all TB cases [105, 106], such that if for some other reason such undiagnosed cases get admitted to the ICU, it becomes apparent for a swift diagnosis and clinical management of such patients with the Xpert MTB/RIF system. Smear microscopy also is incapable of detecting drug resistance. Culture though accurate, requires between 10–1,000 viable mycobacteria per mL of specimen [3] to detect *M. tuberculosis* while having sensitivity of between 80% to 93% and allowing drug susceptibility testing [107] avails results after several weeks impacting on patient treatment regimens and patient management.

Nucleic acid amplification testing requires as few as ~10 to 16 copies of DNA or RNA fragments from a given sample to be sufficient to detect *M. tuberculosis* [108].

Xpert MTB/RIF assay combines, amongst its added advantages, the ability to simultaneously detect *M. tuberculosis* and rifampicin resistance while it also generates results within a short period of time approximately 2 hrs. The many features of this system include sample decontamination, hands-free operation, on-board sample processing and ultra-sensitive hemi-nested PCR.

Xpert MTB/RIF test was first analysed on sputum samples where its evaluation on the diagnostic utility of the system showed that it was simple to use and highly sensitive, having sensitivity of above 95% [44, 46, 47]. As a result, how the test will perform using non-sputum samples is unknown. Information regards Xpert MTB/RIF performance is being reported in different compartments [36, 56, 57].

Friedrich *et al.* [51] in their study evaluated the utility of Xpert MTB/RIF assay for diagnosing pleural TB using pleural fluid and biopsy specimens on 25 patients, where they found sensitivity of 100%. However, this sample size is small to arrive at proper

evaluation results for using Xpert MTB/RIF test, though feasible, but further research needs to be carried out.

This study evaluated the accuracy of Xpert MTB/RIF using tracheal aspirates from mechanically ventilated patients suspected of having TB in a randomised control trial with a cohort of 120 patients in the ICU.

3.2.2. HYPOTHESIS, AIMAND OBJECTIVES

3.2.2.1. Hypothesis

The Xpert MTB/RIF assay is an accurate point of care TB diagnostic tool using tracheal aspirates from mechanically ventilated ICU patients which leads to improved diagnosis in patients suspected of TB.

3.2.2.2. Aim

To determine the performance outcomes of Xpert MTB/RIF assay using tracheal aspirates from TB suspects in the ICU of Groote Schuur Hospital, Cape Town, South Africa vs. smear microscopy and liquid culture techniques for the diagnosis of TB.

3.2.2.3. Objectives

- To determine the diagnostic accuracy of Xpert MTB/RIF assay in smear-positive and -negative patients.
- To determine Xpert MTB/RIF performance in HIV infected and un-infected patients.

3.2.3. MATERIALS AND METHODS

3.2.3.1. Study design

This study was carried out as a single centre randomised controlled trial which was carried out to determine the performance of Xpert MTB/RIF in mechanically ventilated ICU patients suspected of TB recruited at Groote Schuur Hospital, Cape Town, South Africa.

3.2.3.2. Sample size

120 patients were recruited in the parent study but for this particular analysis only 71 patients were used. Statistical analysis was done using a statistical software; OpenEpi (Open Source Epidemiologic Statistics for Public Health, Version 2.3. www.OpenEpi.com) and Graphpad Prism (version 5).

3.2.3.3. Study flow

Tracheal aspirates collected were processed at the Lung Infection and Immunity Unit Laboratory. Xpert MTB/RIF was performed on all tracheal aspirates collected, where a tracheal aspirate of less than 3 ml was collected, 20 mls of saline was added to the tracheal-bronchial tree and aspirated. Clinical and demographic data was recorded, while tracheal aspirates as well as blood, and urine were obtained for later Xpert MTB/RIF, or alternative test analysis.

3.2.3.4. Subject recruitment

All patients admitted to the ICU with suspected pulmonary TB were randomized to the conventional investigation for TB through smear microscopy and culture, and or Xpert MTB/RIF analysis. Baseline data (age), cormobidities (HIV status) were recorded. All patients were tested for HIV and this was done routinely by the admitting unit.

3.2.3.5. Classification of patients

All patients with a positive Xpert MTB/RIF or smear or culture were designated to be definite TB cases, while those who had negative test results for TB and improved on alternative non-tuberculous therapy were non-TB.

3.2.3.6. Laboratory based Methods

Approximately 3 to 15 mls of tracheal secretions was aspirated by means of a sterile-suction catheter attached to the endotracheal tube of the mechanically ventilated patient. Samples collected were sent to the laboratory for routine processing, including Gram stain to assess adequacy of sputum sample and smear microscopy for *M. tuberculosis* and liquid culture (MGIT 960) in all patients. Two to 10 ml of unprocessed tracheal aspirate was stored at -80°C for later analysis.

3.2.3.7. Xpert MTB/RIF assay

The GeneXpert Dx System (Cepheid, Sunnyvale, CA) is an integrated TB diagnostic device that performs sample processing and real-time PCR analysis in a single hands-free step[47]. The Xpert MTB/RIF assay consists of the Xpert MTB/RIF plastic cartridge, containing liquid sample-processing and PCR buffers and lyophilized real-time PCR reagents and the GeneXpert instrument, which controls intra-cartridge fluidics and performs real-time PCR analysis [109].The manual sample pre-treatment steps for the assay involves adding sample reagent buffer to tracheal aspirate samples in a 2:1 ratio respectively and 2 mls of this sample-reagent buffer is then transferred to the sample chamber of the cartridge according to manufacturer's instructions [98]. The cartridge is then inserted in the GeneXpert machine and from this point onwards, all steps are automated. The GeneXpert machine first captures MTB bacilli from the tracheal aspirate sample on a filter membrane where inhibitors and non-specific proteins are then washed from the captured cells with the buffer and finally the captured, washed cells are lysed, such that the released DNA is eluted through the filter membrane. The DNA solution finally gets mixed with dry PCR reagents and thus transferred into the PCR tube for real-time PCR and detection. To eliminate test-to-test contamination, all fluids including amplicons are contained within the disposable cartridge.

3.2.4. RESULTS

3.2.4.1. Patient enrolment and test results

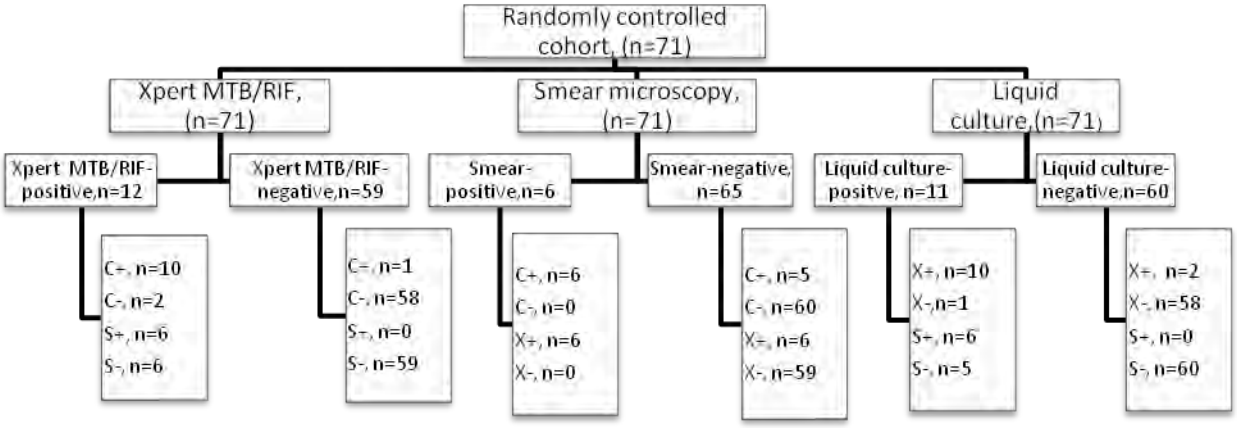


Figure 17: Study flow diagram showing the patients included in the analysis and test results.

Definition of abbreviations: C+; culture-positive patients, C-; culture-negative patients, S+; smear-positive patients, S-; smear-negative patients, X+; Xpert MTB/RIF-positive patients, X-; Xpert MTB/RIF-negative patients.

3.2.4.2. Study population and TB diagnoses

71 patients suspected of pulmonary TB were recruited and consented to participate in the study; Figure 17. Of these 71 patients, 25 (42%) were HIV-infected (Table 11). 11 out of the 71 patients (15%) were tracheal aspirate culture-positive while 60 of the 71 patients (85%) were tracheal aspirate culture-negative for *M. tuberculosis*.

Table 11. Demographic and clinical characteristics.

	Cohort (n=71)	Culture-positive TA (n=11)	Culture-negative TA (n=60)	p-Value
Median Age (IQR)	41.5 (32-54)	28 (19-41)	38.5 (29-52)	
Male gender (%)	41 (58)	7 (64)	34 (57)	0.7
HIV-infected*(%)	25 (42)	4 (44)	21 (41)	0.9
MedianCD4 count (cells/µl) (IQR) if HIV-infected+	158.5 (58-379)	90 (30-190)	158.5 (58-379)	
Previous TB treatment ≠ (%)	24 (36)	3 (30)	21 (37)	0.7
Smoker ¥ (past or current) (%)	21 (32)	4 (44)	17 (30)	0.4

***11 patients were of unknown HIV status (test refused or data missing)**
+one patient with HIV infection was missing CD4 count data
≠4 patients were missing previous TB data
¥6 patients were missing smoking data
TA, tracheal aspirate; TB, tuberculosis
(P-value comparisons are between culture-positive and culture-negative TB patients)

3.2.4.3. Diagnostic accuracy of Xpert MTB/RIF performed on tracheal aspirates

Of the 11 culture-positive TB patients in this cohort, 10 patients had Xpert MTB/RIF-positive tracheal aspirates. Xpert MTB/RIF had a sensitivity of 91% (10/11, 62-98%), while smear microscopy performed on the same tracheal aspirate yielded a sensitivity of 55% (28 - 79%, 6/11), Table 12. However this difference in sensitivity

between Xpert MTB/RIF and smear microscopy (91% vs. 55%) was not significant (10/11 vs. 6/11, p=0.081, Table 12) respectively. 2 of the 60 non-TB patients had Xpert MTB/RIF-positive tracheal aspirate giving a specificity of 97% (88- 99%, 58/60, Table 12) which was similar to smear microscopy which had a specificity of 100% (60/60; 94- 100%; p=0.248). The positive predictive values (PPV) were similar for Xpert MTB/RIF and smear microscopy, 83% (10/12; 55 - 95%) and 100% (6/6; 61 - 100%; p=0.431),respectively, while the negative predictive values (NPV)for the same tests were similar, 98% (58/59; 91 - 100%) and 92% (60/65; 83 - 97%; p=0.146,respectively, Table 12). Co-infection with HIV did not impact on Xpert MTB/RIF assay sensitivity and specificity (p=0.444; p=0.588, respectively) similarly to PPV and NPV (p=0.154; p=0.431) respectively.

3.2.4.4. Xpert MTB/RIF performance in smear-negative cases

Xpert MTB/RIF sensitivity in smear-negative TB patients was found to be 80% (38 - 96%; 4/5) while it also retained a higher specificity of 97 % (89 - 99%; 58/60; Table 12). In HIV-uninfected patients who were smear-negative for TB, the assay had sensitivity of 100% (21 - 100%; 1/1; p=0.667) which was not different from when Xpert MTB/RIF was done in HIV-infected patients having sensitivity of 50% (9 - 91%; 1/2).

3.2.4.5. Accuracy of the Xpert MTB/RIF in HIV co-infected patients.

Amongst HIV-infected patients, Xpert MTB/RIF had sensitivity of 75% (30 - 95%; 3/4), which was not significantly different when the same tracheal aspirate was subjected to smear microscopy yielding sensitivity of 50% (15 - 85%; 2/4; p=0.571), Table 12. Xpert MTB/RIF performance in HIV- uninfected patients had sensitivity of 100% (57 - 100%; 5/5) which was not different from Xpert MTB/RIF performed in HIV-infected patients, p=0.444; Table 12.

Table 12. Accuracy of Xpert MTB/RIF performed on tracheal aspirate, stratified by the HIV status of patients. (Liquid culture-positivity for *M. tb.* serves as a reference standard).

	All patients (n=71)		**HIV-uninfected (n=35)¶**		**HIV-infected (n=25)¶**	
	sens. (95% CI) n/N	**spec.** (95% CI)$^{\beta}$ n/N	**sens.** (95% CI) n/N	**spec.** (95% CI)$^{\beta}$ n/N	**sens.** (95% CI) n/N	**spec.** (95% CI)$^{\beta}$ n/N
Conc. Smear microscopy (%)	55 (28-79) 6/11	100 (94-100) 60/60	80 (38-96) 4/5	100 (89-100) 30/30	50 (15-85) 2/4 (p=0.452)$^{+}$	100 (85-100) 21/21
Xpert MTB/RIF performed on TA (%)	91 (62-98) 10/11 (p=0.081)$^{\text{ł}}$	97 (89-99) 58/60 (p=0.248)$^{\text{ł}}$	100 (57-100) 5/5 (p=0.500)$^{\text{ł}}$	97 (83-99) 29/30 (p=0.500)$^{\text{ł}}$	75 (30-95) 3/4 (p=0.444)$^{+}$ (p=0.571)$^{\text{ł}}$	100 (85-100) 21/21 (p=0.588)$^{+}$
Xpert MTB/RIF performed on TA in smear-negative patients (%)	80 (38-96) 4/5 (p=0.625)$^{\neq}$	97 (89-99) 58/60 (p=0.998)$^{\neq}$	100 (21-100) 1/1	97 (83-99) 29/30 (p=0.998)$^{\neq}$	50 (9-91) ½ (p=0.667)$^{+}$ (p=0.667)$^{\neq}$	100 (85-100) 21/21 (p=0.588)$^{+}$
	PPV (95% CI) n/N	**NPV** (95% CI) n/N	**PPV** (95% CI) n/N	**NPV** (95% CI) n/N	**PPV** (95% CI) n/N	**NPV** (95% CI) n/N
Conc. Smear microscopy (%)	100 (61-100) 6/6	92 (83-97) 60/65	100 (51-100) 4/4	97 (84-99) 30/31	100 (34-100) 2/2	91 (73-98) 21/23 (p=0.459)$^{+}$
Xpert MTB/RIF performed on TA (%)	83 (55-95) 10/12 (p=0.431)$^{\text{ł}}$	98 (91-100) 58/59 (p=0.146)$^{\text{ł}}$	83 (44-97) 5/6 (p=0.600)$^{\text{ł}}$	100 (83-100) 29/29 (p=0.517)$^{\text{ł}}$	100 (44-100) 3/3 (p=0.667)$^{+}$	95 (78-99) 21/22 (p=0.431)$^{+}$ (p=0.641)$^{\text{ł}}$
Xpert MTB/RIF performed on TA in smear-negative patients (%)	67 (30-90) 4/6 (p=0.490)$^{\neq}$	98 (91-100) 58/59 (p=0.998)$^{\neq}$	50 (9-96) ½ (p=0.500)$^{\neq}$	100 (88-100) 29/29	100 (21-100) 1/1 (p=0.667)$^{+}$	95 (78-99) 21/22 (p=0.431)$^{+}$

						(p=0.998)[≠]

Definition of abbreviations: CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value; TA, tracheal aspirate.
¶Excludes 11 patients (who were missing HIV results or did not have HIV data recorded).
βspecificity calculations were based on culture negative samples.
[+]P-values marked with a[+] are for comparisons according to HIV-status.
[ł]P-values marked with a[ł] are for comparisons between Xpert MTB/RIF and smear microscopy.
[≠]P-values marked with a[≠] are for comparisons between Xpert MTB/RIF and Xpert performed in smear-negative patients.

3.2.4.6 **Relationship of Xpert MTB/RIF-generated cycle threshold values with bacterial load**

There was no correlation between Xpert MTB/RIF C_T values and time to positive culture (TTP); (r^2=0.569; p=0.083; Figure 18).

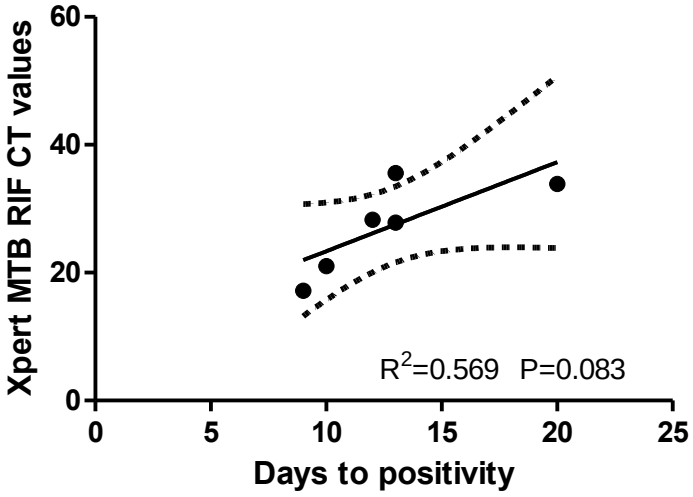


Figure 18: Correlation of tracheal aspirate Xpert MTB/RIF cycle threshold values and Bactec MGIT 960 TTP.

3.2.5. DISCUSSION

Data regarding accuracy of Xpert MTB/RIF when performed on tracheal aspirates is limited. Parameters such as HIV-infection, specimen morphological characteristics and processing are unknown. More often in sputum scarce, and or smear-negative TB cases, sputum induction and or bronchoscopy is used especially in resource limited countries with the bronchoscopy being expensive and requiring specialized personnel such as pulmonologist to perform the procedure. The key findings of this study are as follows: (1) the Xpert MTB/RIF outcompeted smear microscopy, although not significant which can show that when empirical treatment with smear microscopy as the only available rapidly used test in high TB-burdened, resource poor countries, Xpert MTB/RIF maybe overestimated; (2) Xpert MTB/RIF can be used as a rule out test in HIV-uninfected and –infected patients due to the high NPV and PPV which are of great importance in deciding isolation release [110, 111].

In this study of 71 patients, the sensitivity of Xpert MTB/RIF on tracheal aspirates was 91%. Similarly, in a study in France, by Armand *et al*. [112], they reported a sensitivity of Xpert MTB/RIF to be 79% with respiratory specimens which is below our study findings; while in another study conducted in a moderate TB prevalence setting, Laraque *et al.* [113] reported that in smear-positive specimens, their NAAT had a sensitivity of 96%. In a study in France, the investigators reported sensitivity of 90% using Real-time PCR in respiratory specimens, with a PPV of 100% and NPV of 96% [114], while Marlowe *et al*. [115] in their study of the Xpert MTB/RIF in Western USA on 217 specimens, reported a sensitivity of 98% with smear-positive specimens. This shows that our study had the test sensitivity falling within the range exhibited by these commercial NAATs [113-115].

In a meta-analysis of commercial and in-house tests, Pai *et al*., showed that for commercial tests (n=14), they had pooled sensitivity of 56% (46 - 66%) and specificity of 98% (97- 99%), while in-house tests (n=35) had pooled sensitivity and specificity of [76% (67 - 83%); 92% (88 - 95%); respectively)] [116], which shows that our study findings regards sensitivity falls within the range described in their analyses.

In smear-negative patients we reported sensitivity of 80% using the Xpert MTB/RIF, which is similar to other study findings [113, 115] while considerably lesser

sensitivities of between 57% to 67% in other studies [112, 114]. However, this rather modest sensitivity in these studies, might be attributable to differences in decontamination processes, cross contamination, inhibition sampling error, quality of the reference standard and a mixture of respiratory and other specimens in studies [117-120].

We reported an additional two patients who were Xpert MTB/RIF-positive, culture-negative and such patients have been reported to be true representatives of TB cases in a previous study [48].

Some of the limitations of this study are that we didn't capture specimen specific factors such as viscosity and or appearance (bloody or not) which could possibly interfere with Xpert MTB/RIF assay, i.e. the viscosity of the tracheal aspirate even after addition of the sample buffer may not be completely homogenized and thus interfere with the Xpert MTB/RIF system in harvesting bacilli nucleic acid. The other likely parameters might be specimen processing such as centrifugation which has been reported in previous studies to otherwise increase sensitivity [36, 56, 57] due to concentration and sedimentation of the test material which we did not look at in our study.

Conclusion

The Xpert assay on tracheal aspirates is feasible, and can be used as a rule-out TB diagnostic test, and there is potential to increase sensitivity of the assay by optimizing on sample preparation such as collection and processing (centrifugation) of tracheal aspirates.

4. CHAPTER 4

DIAGNOSTIC ACCURACY OF THE XPERT MTB/RIF USING CEREBRO-SPINAL FLUID (CSF) IN DIAGNOSING TB MENINGITIS (TBM).

ABSTRACT

Background

TB-meningitis (TBM) is difficult to diagnose with the available conventional methods. The accuracy of Xpert when performed on cerebral spinal fluid (CSF) of patients suspected of TBM remains unclear. This study evaluated the accuracy of Xpert in diagnosing TBM.

Methods

In this study, 235 South African patients suspected to have TBM had lumbar puncture done. Approximately 15 ml of CSF was obtained and where appropriate, 1 ml of uncentrifuged CSF was used for Xpert testing, and or when available, 3 ml of centrifuged CSF was used for Xpert analysis. Liquid culture from a specimen collected at the same time was used as the reference standard for definite TB. For each diagnostic assay and fluid tested, we evaluated diagnostic accuracy according to HIV status and smear microscopy status, when these data were available.

Results

Of the 152 evaluable patients, 41 had definite TBM, 54 probable TBM and 57 non-TBM. Of the definite TBM patients, Xpert MTB/RIF significantly outperformed smear microscopy having sensitivity [49% (33-64%) versus 3% (1-15%); **$p \leq 0.001$**, respectively]. In HIV-coinfected patients, centrifugation of CSF resulted in a significant increase in sensitivity of Xpert MTB/RIF test, 100% (68-100%; **$p=0.01$**) than uncentrifuged CSF, 53% (37-69%). There was no correlation between Xpert MTB/RIF C_T values and TTP, $p=0.45$.

Conclusion

Xpert MTB/RIF has more rule-in potential for TBM diagnosis in HIV-infected patients when centrifuged CSF specimens are used.

Research Outputs

Vinod B. Patel, Grant Theron, Laura Lenders, ***Brian Matinyenya****, Cathy Connolly, Ravesh Singh, Yacoob Coovadia, Thumbi Ndung'u, Keertan Dheda (2013). Diagnostic accuracy of quantitative PCR (Xpert MTB/RIF) for tuberculous meningitis in a high burden setting: a prospective study.* PLoS Med, 2013. **10**(10): p. e1001536.

Grant Theron, Jonny Peter, Greg Calligaro, Richard Meldau, Colleen Hanrahan, Hoosain Khalfey, ***Brian Matinyenya****, Tapuwa Muchinga, Liezel Smith, Shaheen Pandie, Laura Lenders, Bongani M. Mayosi, Vinod Patel & Keertan Dheda (2014). Determinats of PCR performance (Xpert MTB/RIF), includng bacterial load and inhibition, for TB diagnosis using specimens from different body compartments. Sci Rep, 2014.* ***4****: p. 5658.*

Vinod B. Patel, Cathy Connolly, Ravesh Singh, Laura Lenders, ***Brian Matinyenya****, Grant Theron, Thumbi Ndung'u, Keertan Dheda (2014). Comparison of Amplicor and GeneXpert MTB/RIF Tests for Diagnosis of Tuberculous Meningitis. doc10.1128/JCM.01235-14.*

4.1. INTRODUCTION

TB remains a significant cause of morbidity and mortality in Sub-Saharan Africa, especially when HIV co-infection is involved [121]. However, up to 40% of co-infected patients have extrapulmonary tuberculosis (EPTB) while, 10% of such patients have TBM [122]. TBM patients require longer periods of hospitalisation, and in the process such patients consume and diminish quite a lot of health-care resources for their management [56].

Available diagnostic platforms such as smear microscopy perform dismally in TBM, with one study reporting sensitivities of less than 20% [94]. Liquid culture techniques such as the mycobacterial growth indicator tube (MGIT; Bactec) and the mycobacterial drug susceptibility assay (MODS) culture aid in improved sensitivity over solid culture up to about a sensitivity of 60% [123]. However the time period (approximately 4 weeks) it takes to give back a positive result limits its use in confirming of disease.

In a meta-analysis by Morgan *et al.* [124], line probe assays such as the INNO-LiPA Rif. TB kit (Innogenetics, Zwijndrecht, Belgium) had sensitivity greater than 95%, showing that such NAAT's had increased diagnostic potential; however, due to their requirements for technical expertise, being expensive and prone to contamination, such a system becomes unsuitable for implementation in resource-limited settings.

Molecular techniques such as the Cobas Amplicor MTB (Roche Diagnostics, Indianapolis, IN) has been widely used for the detection of *M. tuberculosis* in respiratory samples while problems have been reported particularly in non-respiratory samples such as the presence of inhibitor enzymes and contamination [125]. Sensitivity is often variable and offer little advantage over smear microscopy [126].

Thus as a result, the current diagnostic methods are somewhat limited in sensitivity and applicability, while also due to the paucibacillary nature of TBM and the requirement for larger volumes of CSF that are rather difficult to obtain in cases of children makes such techniques limited in diagnosing TBM [127].

Comparisons of TBM diagnostic studies are limited by the lack of a clear reference standard and low study numbers. Xpert MTB/RIF's value has not previously been evaluated in TBM and also factors such as the impact of sample volume, sample

processing (centrifuged and uncentrifuged), LOD, effect of CSF-related PCR inhibition, and the relationship between CSF bacterial load and Xpert MTB/RIF cycle threshold (C_T) values have not been determined [56].

This study evaluated the accuracy of Xpert MTB/RIF in an unselected cohort of patients with suspected TBM.

4.2. HYPOTHESIS, AIM AND OBJECTIVES

4.2.1. Hypothesis

The Xpert MTB/RIF assay is an accurate point of care TB diagnostic tool using CSF as a bio-specimen which will lead to improved diagnosis in patients suspected of TBM.

4.2.2. Aim

To determine the performance outcomes of Xpert MTB/RIF assay on CSF from TBM suspected patients at a tertiary hospital in KwaZulu-Natal, Durban, South Africa vs. smear microscopy and liquid culture techniques for the diagnosis of TBM.

4.2.3. Objectives

- To determine the diagnostic accuracy of Xpert MTB/RIF assay in smear-negative and smear-positive patients.
- To determine Xpert MTB/RIF performance in HIV-infected and -uninfected patients.
- To determine the performance of Xpert in uncentrifuged and centrifuged specimens.

4.3. MATERIALS AND METHODS

4.3.1. Study Design

This study was prospectively carried out to determine the performance of the Xpert MTB/RIF assay in patients suspected of having TBM who were recruited at local district hospitals and referred to the Inkosi Albert Luthuli Central Hospital in KwaZulu Natal, Durban, South Africa. Xpert MTB/RIF testing was performed at the Lung Infection and Immunity Unit, Cape Town, and Xpert MTB/RIF results were not used for patient management.

4.3.2. Sample size

In total, 235 consecutive patients with suspected meningitis were prospectively recruited.

4.3.3. Study Flow and Subject recruitment

Patients with a meningitic illness who were referred from district hospitals were recruited. Detailed patient and laboratory specific information was recorded. Written informed consent was obtained from the patient or close relatives in cases where patients were unable to provide consent, and a lumbar puncture was clinically indicated, the Head of Department of Neurology was approached for consent [56, 128] . Eligible patients who signed the informed consent form had their medical history taken and clinically assessed, underwent a computerised tomography (CT) scan to exclude contraindications to a lumbar puncture, and had blood drawn for routine tests including a serum fluorescent treponemal antibody test, a venereal disease research laboratory test, HIV enzyme-linked immunosorbent assay, and a CD4 count [56]. Samples were collected before the patient started TB treatment. All results were recorded in the Case Report Form (CRF), which was entered on an electronic database. Study participants with incomplete CRF's were withdrawn and a new study subject enrolled instead. Statistical analysis was done using a statistical software; OpenEpi (Open Source Epidemiologic Statistics for Public Health, Version 2.3. www.OpenEpi.com) and Graphpad Prism (version 5).

4.3.4. Classification of patients

Patient classification was based on standardized published diagnostic criteria, as definite TBM if the CSF *M. tuberculosis* culture and or Amplicor PCR were positive or non-TBM (alternate diagnosis confirmed and response to therapy documented in the absence of anti-TB treatment) [129, 130].

4.3.5. Lab based methods

Approximately 15 ml of CSF, obtained by lumbar puncture was processed for the following tests: microscopy (Gram strain and fluorescent staining for acid-fast bacilli [auramine]); bacterial culture; *Mycobacterium tuberculosis* culture (BACTEC MGIT 960; BD); fungal culture, cryptococcal latex agglutination test; Roche Amplicor *Mycobacterium tuberculosis* PCR test (Roche Diagnostics System) (Amplicor PCR); routine chemistry (protein, glucose and chloride); viral PCR for cytomegalovirus; varicella zoster virus, and herpes simplex; venereal disease research laboratory test; fluorescent treponemal antibody test; and test for cysticercus antibodies , while an uncentrifuged specimen and centrifuged sample of CSF was biobanked for Xpert MTB/RIF analysis if there was sufficient volumes available [56].

4.3.5.1. Amplicor PCR

An independent lab processed 197 samples using the Amplicor PCR kit for the detection of *M. tuberculosis* as per manufacturer's protocol. DNA was extracted from 0.5 ml of CSF using the Roche Magna Pure automated DNA extraction system using the DNA high performance kit where the extracted DNA was then amplified using biotinylated primers KY18 and KY75 as described in the Amplicor PCR kit protocol [56]. PCR products were detected by the Cobas Amplicor analyser according to the kit protocol.

4.3.5.2. Xpert MTB/RIF

This test is a fully automated real-time, hemi-nested PCR system [55], developed to simultaneously detect *M. tuberculosis* and rifampicin resistance in a single-use cartridge hands free-step [44, 47]. In the initial period of the study, only 1 ml of uncentrifuged CSF was obtained for Xpert MTB/RIF testing from 149 patients suspected of TBM. Thereafter, so as to assess the impact of centrifugation, a 3 ml centrifuged pellet was obtained from 59 patients suspected of TBM and then re-

suspended in 1 ml of phosphate-buffered saline (PBS). In this latter period, if enough CSF was available, both a 1 ml uncentrifuged and 3 ml centrifuged sample were evaluated i.e. either a 1 ml or 3 ml sample or both was processed for Xpert MTB/RIF in patients suspected of TBM [56]. Batched, archived (-70°C) uncentrifuged samples (n=149) and centrifuged and uncentrifuged (n=59) were processed at the Lung Infection and Immunity Unit (Dept of Medicine, Groote Schuur Hospital, University of Cape Town) for Xpert MTB/RIF analysis. Lab technicians performing the CSF culture, Xpert MTB/RIF assay and Amplicor were blinded to all participant details. Samples for Xpert MTB/RIF test were prepared according to the manufacturer's instructions [98]. Frozen, unprocessed samples were immediately processed with the CSF-sample reagent mixture shaken and incubated at room temperature for a total of 15 minutes with a second shake at 10 minutes. 2 ml of the digested mixture was then transferred to the Xpert MTB/RIF cartridge [56].

4.4. RESULTS

4.4.1. Study population and TB diagnoses

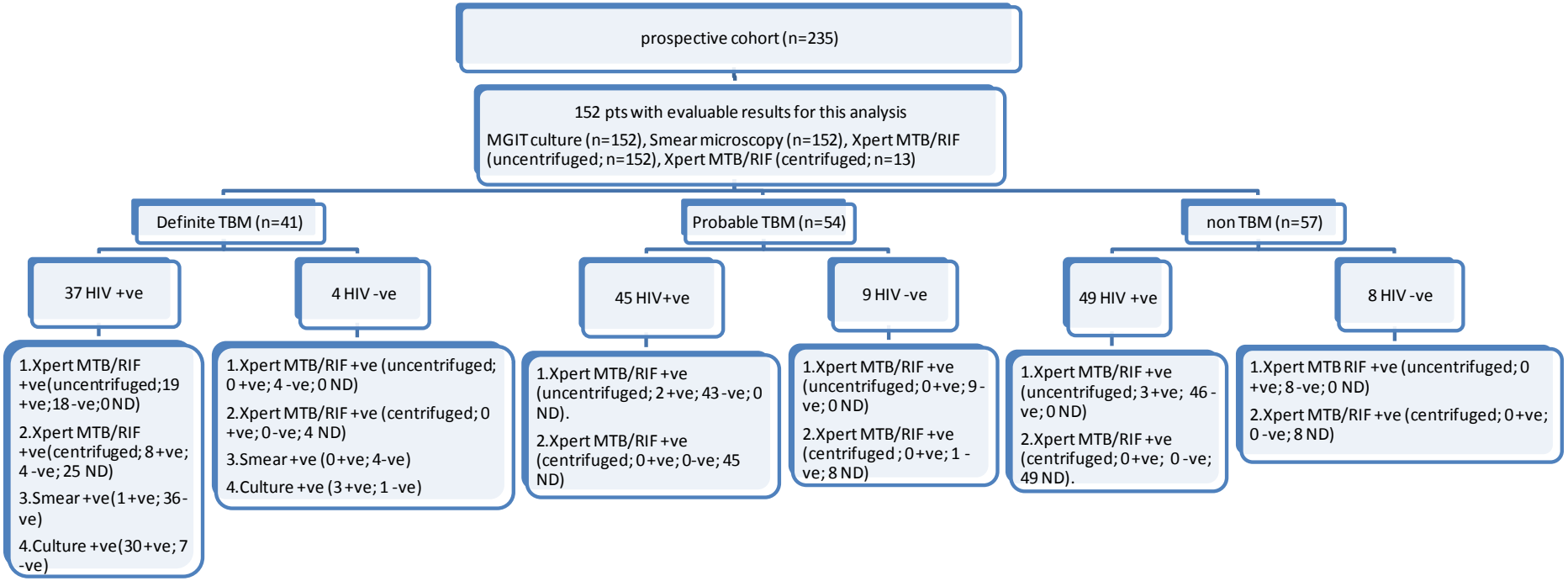


Figure 19: Summary flow chart showing patients included in the analysis and diagnostic testing performed.

Definition of abbreviations. MGIT, BACTEC MGIT 960; ND, not done; +ve, positive; -ve, negative; pts, patients.

235 patients with suspected TBM were recruited in this study. However for this particular analysis, 152 patients had evaluable results, Figure 19. Of the 152 patients included, 131 (86%) were HIV-infected, while 21 (14%) were HIV-uninfected, Figure 19. 41 of the 152 patients (27%) were definite TBM cases, and 57 (38%) of the 152 patients were non-TBM, Table 13.

Table 13. Demographic and Clinical characteristics

Demographic /Clinical Characteristics	Definite TBM (n=41)	Non TBM (n=57)	P-value
Median age/ yrs	26.5	43	0.86
Male	18 (44)	20 (35)	0.39
Female	23 (51)	37 (65)	0.39
Ethnic group			
Black	40 (98)	56 (98)	0.84
Mixed	1 (0.03)	-	NA
Indian		1 (0.02)	
HIV infected pts	37 (90)	49 (86)	0.55
HIV uninfected pts	4 (0.1)	8 (14)	0.55
Median CD4[ł] count (cells/µl) if HIV +ve	75.5	69.5	0.07
Previous TB[≠] (%)	8 (20)	21 (36.8)	0.06

NA, not applicable; TB, tuberculosis; +ve, positive.
[ł]nine patients with HIV infection were missing CD4 count.
[≠]six patients were missing previous TB data.

4.4.2. Diagnostic accuracy of Xpert MTB/RIF performed on CSF

Out of the 35 definite TB (culture-positive or Amplicor-positive) patients in this cohort, 17 had Xpert -positive CSF. Xpert had sensitivity of [49% (33 - 64%); p≤0.001; Table 14] which as significantly higher than smear microscopy performed on the same CSF, having sensitivity of 3% (1 - 15%). 7 of the 117 non TB cases (culture-negative or Amplicor-negative) had Xpert -positive CSF with a specificity of 94% (110/117; 88- 97%) equivalent to that of smear microscopy [100% (11/117; 97 - 100%); p=0.785, Table 14]. The positive predictive value (PPV) of Xpert was 71% (51 - 85%; 17/24) which was similar to that of smear microscopy, 100% (21 - 100%; 1/1; p=0.720), while the negative predictive value (NPV) of Xpert, 86% (110/128; 79 - 91%; p=0.072) was similar to that of smear microscopy, 78% (117/151; 70 - 83%).

4.4.3. Impact of CSF centrifugation on Xpert MTB/RIF performance

CSF centrifugation resulted in an increase in sensitivity of Xpert from 49% (17/35; 33 - 64%) to 94% (17/18; 74 - 99%), however this increase in sensitivity was not significant, p=0.008, Table 14.

4.4.4. Accuracy of Xpert MTB/RIF in HIV coinfected patients

In HIV-infected patients, centrifugation of CSF resulted in the sensitivity of Xpert to be 100% (8/8; 68 - 100%; p=0.014, Table 14) which was significantly different from Xpert performance in uncentrifuged CSF (53%; 17/32; 37 -69%). The PPV of Xpert on centrifuged CSF was 94% (17/18; 74 - 99%; p=0.065) which was similar to Xpert performance on uncentrifuged CSF, 71% (17/24; 51-85%). NPV of Xpert in centrifuged vs. uncentrifuged CSF was similar (86% vs. 86%, p=0.919), respectively.

4.4.5. Xpert MTB/RIF performance in smear-negative patients

In smear-negative patients Xpert MTB/RIF had a sensitivity of 49% (17/35; 33- 64%) and a specificity of 94% (110/117; 88 - 97%), Table 14. The PPV of Xpert MTB/RIF in smear-negative patients vs. Xpert in unprocessed CSF was the same (71% vs. 71%), which was similar as well in their NPV's (86% vs. 86%).

4.4.6. Diagnostic accuracy of Xpert MTB/RIF performed on CSF

Table 14. Accuracy of Xpert MTB/RIF performed on CSF and stratified by HIV status of patients. (Liquid culture positivity for TBM serves a reference standard).

	All patients (n=152)		**HIV-uninfected (n=21)**		**HIV-infected (n=131)**	
	sens. (95% CI) n/N	**spec.** (95% CI) n/N	**sens.** (95% CI) n/N	**spec.** (95% CI) n/N	**sens.** (95% CI) n/N	**spec.** (95% CI) n/N
Conc. smear microscopy (%)	**3** (1-15) 1/35	**100** (97-100) 111/117	**0**	**100** (82-100) 18/18	**3** (1-16) 1/32	**100** (96-100) 99/99
Amplicor (%)	**42** (26-59) 13/31	**93** (87-96) 108/116	0	**95** (75-99) 18/19	**46** (30-64) 13/28	**93** (86-97) 91/98
MTB/RIF performed on uncentrifuged CSF (%)	**49** (33-64) 17/35 **(p≤0.001)**[ł]	**94** (88-97) 110/117 (p=0.785)[ł]	**0**	**100** (82-100) 18/18	**53** (37-69) 17/32 **(p=0.000005)**[ł]	**93** (86-97) 92/99 (p=0.300)[+] (p=0.07)[ł]
MTB/RIF performed on uncentrifuged CSF in smear negative patients (%)	**49** (33-64) 17/35 (p=0.998)[≠]	**94** (88-97) 110/117	0	**100** (82-100) 18/18	**50** (34-66) 16/32 (p=0.809)[≠]	**48** (32-65) 15/31 **(p=0.0009)**[+] **(p=0.0000003)**[≠]
MTB/RIF performed on a re-suspended pellet (centrifuged CSF) (%)	**94** (74-99) 17/18 (p=0.008)[β]	**86** (49-97) 6/7 (p=0.446)[≠]	**67** (21-94) 2/3	**100** (44-100) 18/18	**100** (68-100) 8/8 **(p=0.014)**[β] (p=0.273)[+]	**50** **(10-91)** 1/2 (p=0.100)[+] **(p=0.020)**[β]
	PPV (95% CI) n/N	**NPV** (95% CI) n/N	**PPV** (95% CI) n/N	**NPV** (95% CI) n/N	**PPV** (95% CI) n/N	**NPV** (95% CI) n/N
Conc. smear microscopy (%)	**100** (21-100) 1/1	**78** (70-83) 117/151	**0**	**86** (65-95) 18/21	**100** (21-100) 1/1	**76** (68-83) 99/130 (p=0.351)[+]

Amplicor (%)	**62** (41-79) 13/21	**86** (79-91) 108/126	**0**	**86** (65-95) 18/21	**65** (43-82) 13/20	**86** (78-91) 91/106
MTB/RIF performed on uncentrifuged CSF (%)	**71** (51-85) 17/24 (p=0.720)[ł]	**86** (79-91) 110/128 (p=0.072)[ł]	**0**	**100** (82-100) 18/18 (p=0.146)[ł]	**71** (51-85) 17/24 (p=0.720)[ł]	**86** (78-91) 92/107 (p=0.083)[+] (p=0.058)[ł]
MTB/RIF performed on uncentrifuged CSF in smear negative patients (%)	**71** (51-85) 17/24 (p=0.998)[≠]	**86** (79-91) 110/128 (p=0.998)[≠]	**0**	**86** (65-95) 18/21 (p=0.146)[≠]	**50** (34-66) 16/32 (p=0.129)[≠]	**48** (32-65) 15/31 (p=0.07)[+] **(p=0.00004)[≠]**
MTB/RIF performed on a re-suspended pellet of CSF (%)	**94** (74-99) 17/18 (p=0.065)[β]	**86** (49-97) 6/7 (p=0.919)[β]	**100** (32-100) 2/2	**75** (30-95) 3/4 (p=0.182)[β]	**89** (56-98) 8/9 (p=0.818)[+] (p=0.330)[β]	**100** (21-100) 1/1 (p=0.800)[+] (p=0.861)[β]

CSF, Cerebro spinal fluid; NPV, negative predictive value; PPV, positive predictive value; TB, tuberculosis.

[+]P-values marked with a[+] are for comparisons according to HIV-status.

[ł]P-values marked with a[ł] are for comparisons between Xpert MTB/RIF and smear microscopy.

[≠]P-values marked with a[≠] are for comparisons between Xpert MTB/RIF and Xpert performed in smear-negative patients.

[β]P-values marked with a[β] are for comparisons between Xpert MTB/RIF centrifuged and Xpert MTB/RIF uncentrifuged

0 – means incalculable, as there were too few HIV-uninfected patients to generate reliable accuracy data

4.4.7. Xpert MTB/RIF quantitative information and bacterial load analysis

There was no correlation between Xpert MTB/RIF C_T CSF values and time to positive culture (TTP), (R^2=0.083; P=0.451; Figure 20).

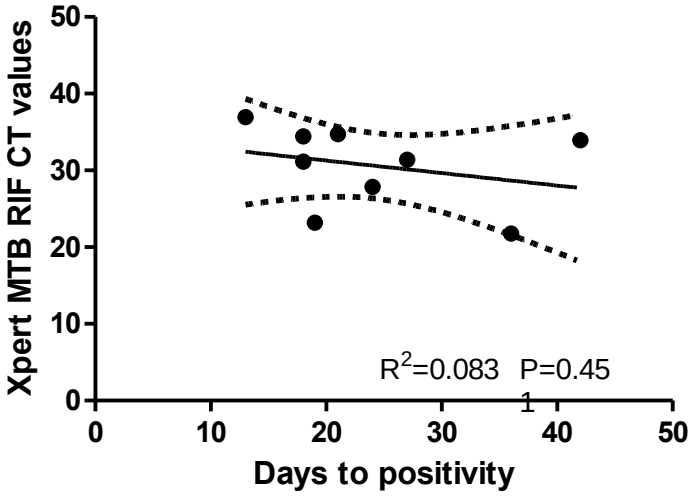


Figure 20: Relationship between Xpert MTB/RIF-generated cycle threshold (C_T) values and bacterial load in CSF.

4.4.8. DISCUSSION

Xpert MTB/RIF evaluation in biological fluids other than sputum is of paramount importance as it guides its usage and administration in managing patients suspected of having TB. The key findings of this study included: (1) Xpert is likely an important rule-in test for the diagnosis of TBM in patients with HIV where it outperformed smear microscopy, which is relevant in TBM patient management and treatment outcomes; (2) sample processing, such as centrifugation of the CSF improved sensitivity particularly to HIV-infected patients in this analysis.

Limited data exists in relation to Xpert MTB/RIF performance in TBM [131]. Available studies evaluating Xpert MTB/RIF on TBM either have too few CSF data, such as in a study by Hillemann *et al. [52]*,where nineteen CSF samples were evaluated as part of a bigger study of extrapulmonary samples and had a specificity of 100% with a combined sensitivity of 77%; while also in a survey in Greece by Ioannidis *et al.* [132], the sensitivity of Xpert MTB/RIF was 100% but of note is the fact that CSF constituted about 17% of the total constituent of extrapulmonary specimens showing the smaller number of samples at hand. Tortoli *et al.* [55] showed that Xpert MTB/RIF test had a sensitivity of 85%, however the study had a lower number of culture-positive confirmed cases and the fact that the study was performed in a non-TB endemic country suggest otherwise regards its usefulness in a high TB endemic area even though the study cohort is predominantly high with 1476 specimens. Thus all of these studies [52, 55, 131, 132], have small numbers of microbiologically proven TBM cases ranging from 0 to 23, where CSF related data constitutes a smaller percentage of the total constituent of samples being studied as they are mainly laboratory based evaluations of EPTB samples in low TB burden countries.

There was no correlation between Xpert MTB/RIF C_T values and TTP in this study, which is different from findings in a sputum based study by Theron *et al.* [133], where they found that sputum bacillary load was elevated in tuberculosis patients who were less immunosuppressed.

In this study in HIV-infected patients having centrifuged CSF samples, Xpert MTB/RIF had a high sensitivity of 100%; highly suggestive that in areas with high HIV rates, it might be an improved new standard of diagnosis and care for TBM. In a

study carried out in South Africa [36] , it was reported that centrifugation of urine improved the diagnostic yield when Xpert MTB/RIF test was used in HIV-infected, sputum scarce patients. This is in accordance with our findings when a similar amount of CSF was used resulting in sensitivity of 100%.

Centrifugation works in CSF possibly due to the fact that the fluid is always readily available and is sterile and isn't made in response to an infection so in cases of infection the bacilli is within the fluid such that when one collects the CSF and concentrates it, chances are highly likely that the bacilli or test material is sedimented and pelleted, the same with urine. However, the same can't be said for BALF, a possible explanation being that the bugs are out-diluted as approximately 300 ml of saline is added to the compartment and at least 150 ml of fluid is retained, so in the process some bacilli might be lost such that even when centrifugation is done there will be too few if any bacilli available for pelleting.

Xpert MTB/RIF in this study performed with a higher sensitivity compared to other commercially available tests. In a study by Causse *et al.* [50],in Spain, Xpert MTB/RIF sensitivity was reported to be 95% against 78% for the Cobas TaqMan MTB, where in another survey in Germany, the Cobas Amplicor MTB assay had sensitivity of 66% [125]. Jonsson *et al.* [134], in their study with a cohort of 154 samples of CSF they analyzed using the Cobas Amplicor MTB assay, sensitivity and specificity was found to be 56% and 97%, respectively. All this information is suggestive that our findings prove that the Xpert MTB/RIF test performed considerably much better than the commercially available tests presented herein. However, a likely possible explanation to this improvement in performance was likely probably due to the CSF processing (centrifugation) which is likely to concentrate and sediment bacilli and rather technical modalities such as efficient extraction and fractionation protocols which are automated, thus improving amplification [56], while also, the fact that our study was carried out in a higher HIV prevalent setting, makes it a possibility we had higher bacterial loads in these patients.

Some of the limitations of the study are that it was undertaken in a highly TB endemic area with high HIV co-infection such that its performance might be over emphasized such that it needs to be evaluated in other regions of low TB prevalence so as to properly document its use in diagnostics and patient management. Also, the study had

very few numbers of HIV-uninfected patients (less than 25) such that comparisons in this group were not possible and that it would be important to know how the test performs in this group. The study also shows that the test is useful in determining that a patient has TBM, while it omits on how useful the test is in determining that a patient doesn't have TBM necessitating the need for further research into improving sensitivity of the assay.

Conclusion

The study suggests that the Xpert MTB/RIF has more rule-in potential and is a good diagnostic test of TBM in HIV-infected patients in TB-endemic settings, when centrifuged CSF specimens are used.

5. CHAPTER 5

DISCUSSION AND OVERALL CONCLUSIONS

5.1. THE ACCURACY OF LAMP IN TB DETECTION ON SPUTUM

The use of the LAMP test to diagnose TB suspects within primary health care facilities and microscopy centres in high burden settings seem to be feasible, though further development is required to improve on the stability and sensitivity of the assay. The assay is a promising rule-in test but specificity remains sub-optimal at 91%. Use of the LAMP test in a targeted approach in cases of HIV co-infection did not impact on the sensitivity and specificity of the LAMP test i.e. the assay performed the same in these sub-groups of patients (70%; in HIV-infected patients vs. 85% in HIV-uninfected patients; p=0.162). Those patients that were LAMP-positive had a significant shorter time to culture-positivity than the LAMP-negative patients. The LAMP test does not have incremental value over smear microscopy though it aids in improved case detection. The assay had modest sensitivity, 77%, which might be improved in future further studies probably through assessing the performance on non-sputum samples (CSF, BALF, urine etc), and also maybe through varying specimen morphological characteristics such as viscosity, appearance (bloody or not), specimen processing (centrifugation), which might possibly lead to improved sensitivity of the test leading to increased TB diagnostic potential in high burden settings. However, it should be noted that these are preliminary data and the test, though feasible, is not yet ready for use given the high false-positive error rate for use in the clinical diagnosis of TB.

5.2. THE XPERT MTB/RIF ASSAY ON BALF IN THE DETECTION OF TB

The Xpert MTB/RIF assay is a promising rule-in test having a specificity of 96% when performed on BALF from prospective, sputum-scarce, and or smear-negative TB suspects within a high burden setting. Centrifugation of the BALF increased the sensitivity of the assay compared to uncentrifuged BALF, though not significant.

However, bronchoscopy is not yet widely available in marginalized settings where the burden of HIV, sputum-scarce and or smear-negative TB is highest. Our study has more relevance and gives guidance in low incidence settings, where bronchoscopy is readily available. Larger multicentre studies are necessary to properly evaluate and document whether such diagnostic tests such as Xpert MTB/RIF can be used especially given the underperformance of smear microscopy, while also given the lack of readily available expertise in marginalized settings, sputum induction can be assessed to determine the performance outcomes of Xpert MTB/RIF testing on sputum-scarce and or smear-negative TB patients.

5.3. XPERT MTB/RIF ASSAY ACCURACY USING TRACHEAL ASPIRATES TO DIAGNOSE TB

Mechanically ventilated patients subjected to testing by Xpert MTB/RIF in a single randomized control trial had the test having an overall sensitivity of 91% outcompeting smear microscopy which thus reduces empirical treatment of patients, while also having a potential on drug susceptibility testing and accelerated turn-around time especially in a high burden setting like where our study was undertaken. As a result of the predominantly high NPV (98%) and PPV (83%), Xpert in this instance can be a useful rule-out test which is important in deciding isolation release. HIV infection did not impact on the diagnostic accuracy of the Xpert MTB/RIF. Of the patients with positive Xpert MTB/RIF results, 6/12 were smear-negative, and thus Xpert MTB/RIF increased the diagnostic yield by 50%. It would be interesting to look at specimen specific factors such as viscosity, salt concentration and appearance (bloody or not) especially for tracheal aspirates on their influence on the test as salts, proteins and or cellular debris are commonly found in non-sputum samples, while also sample processing (centrifugation; can enrich the cellular debris and salts mentioned herein) which can interfere with the amplification process and thus inhibit PCR leading to unreliable and rather misleading results.

5.4. XPERT MTB/RIF ACCURACY USING CSF TO DIAGNOSE TBM

Xpert MTB/RIF testing has more rule-in potential for TBM diagnosis in a targeted population of HIV-infected patients having specificity of 86%. The suboptimal sensitivity of Xpert MTB/RIF on CSF might be as a result of the presence of inhibitors, and the paucibacillary samples which were below the detection threshold of the assay. However, the rather high sensitivity of 100% when centrifuged samples from HIV-infected patients were tested, suggest that the test in higher burden settings coupled with HIV co-infection poses as a new standard of care in diagnosing TBM. There were too few HIV-uninfected patients (n=4) in this cohort of definite TB patients which might lead to misleading results on the performance outcome of the test such that we were unable to meaningfully compare this group. Studies should be undertaken in different geographical settings of low TB incidence, in HIV-uninfected populations and coupled with varying the sample processing methods (CSF volume, centrifugation etc) so as to clarify the performance outcomes and the diagnostic accuracy of Xpert MTB/RIF in patients in such settings.

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